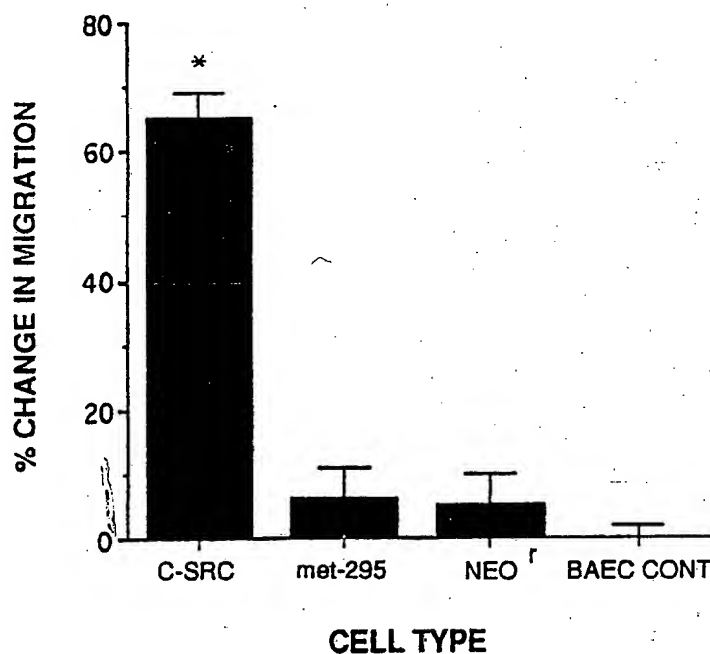


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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US93/00445 <b>(22) International Filing Date:</b> 5 January 1993 (05.01.93) <b>(30) Priority data:</b> 07/820,011      6 January 1992 (06.01.92)      US <b>(71) Applicant:</b> YALE UNIVERSITY [US/US]; 451 College Street, New Haven, CT 06510 (US). <b>(72) Inventors:</b> BELL, Leonard ; 59 Tumblebrook Road, Woodbridge, CT 06525 (US). MADRI, Joseph, A. ; 221 West Pond Road Extension, North Brandford, CT 06471 (US). WARREN, Stephen, L. ; 514 Treat Lane, Orange, CT 06477 (US). LUTHRINGER, Daniel, J. ; 149 North Wilamon Drive, Beverly Hills, CA 90211 (US).			<b>(74) Agent:</b> KLEE, Maurice, M.; 1951 Burr Street, Fairfield, CT 06430 (US). <b>(81) Designated States:</b> AU, BR, CA, FI, HU, JP, KR, NO, NZ, RU, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). <b>Published</b> <i>With international search report.</i>

**(54) Title:** GENETICALLY ENGINEERED ENDOTHELIAL CELLS**(57) Abstract**

Genetically engineered endothelial cells which exhibit enhanced cell migration, enhanced urokinase-type plasminogen activator (u-PA) activity, reduced mononuclear cell (e.g., monocyte) adhesion, and reduced fibronectin production are provided. The cells are modified by incorporation of the coding sequence for the c-src gene so that the cells express elevated levels of the tyrosine kinase protein, pp60<sup>c-src</sup>.

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GENETICALLY ENGINEERED ENDOTHELIAL CELLS

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10 reasonable terms as provided for by the terms of Grants Nos. 5K11-HL02351, RO1-HL28373, and 9T32 DK07556 awarded by The National Institutes of Health, Bethesda, Maryland.

FIELD OF THE INVENTION

15

This invention relates to genetically engineered endothelial cells and, in particular, to genetically engineered endothelial cells which exhibit enhanced migration, enhanced plasminogen activator activity, reduced mononuclear cell (e.g., monocyte) adhesion,  
20 and reduced fibronectin production.

BACKGROUND OF THE INVENTION

25

Endothelial cells are specialized cells which form the lining of the heart and the blood vessels. Because of their direct contact with the circulating blood, a number of proposals have been made to  
25 genetically engineer these cells and use them as "in vivo" drug delivery systems. See, for example,

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Culliton, B. J. 1989. "Designing Cells to Deliver Drugs," Science. 246:746-751; and Zwiebel, J. A., S. M. Freeman, P. W. Kantoff, K. Cornetta, U. S. Ryan, and W. F. Anderson. 1989. "High-Level Recombinant  
5 Gene Expression in Rabbit Endothelial Cells Transduced by Retroviral Vectors," Science. 243:220-222. (transfer of a human adenosine deaminase gene and a rat growth hormone gene to aortic endothelial cells using a retroviral vector and  
10 demonstration of the secretion of rat growth hormone from such cells after seeding onto a synthetic vascular graft).

Endothelial cells are known to play an important role in the pathogenesis of atherosclerotic plaques,  
15 as well as in the success or failure of various surgical procedures, including vascular stent implantation, coronary angioplasty, and coronary bypass surgery using autologous veins or arteries or synthetic materials, such as, dacron or expanded  
20 polytetrafluoroethylene.

Endothelial cells affect both the disease process and efforts to reconstruct damaged vessels because, among other things, they can: 1) alter the thrombogenic properties of the blood vessel wall, 2)  
25 modulate smooth muscle cell proliferation and migration, and 3) affect vascular smooth muscle tone through multiple pathways including the renin-

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angiotensin system (i.e., the system wherein the proteolytic enzyme renin cleaves two amino acids from angiotensin I to produce the hypertensive agent angiotensin II).

5           With regard to their interaction with the renin-angiotensin system, investigators have demonstrated in vitro that many of the constituents of that system, including angiotensinogen, renin, angiotensin-converting enzyme, and angiotensin II  
10 receptors, are contained within endothelial cells thus forming an autocrine angiotensin system. See Lilly, L. S., R. E. Pratt, R. W. Alexander, D. M. Larson, K. E. Ellison, M. A. Gimbrone, and V. J. Dzau. 1985. "Renin expression by vascular  
15 endothelial cells in culture," Circ. Res. 57:312-318; Caldwell, P. R. B., B. C. Seegal, and K. C. Hsu. 1976. "Angiotensin-converting enzyme: vascular endothelial localization," Science (Wash. DC).  
191:1050-1051; Ryan, U. S., J. W. Ryan, C. Whitaker, and A. Chiu. 1976. "Localization of angiotensin  
20 converting enzyme (kininase II). II. Immunocytochemistry and immunofluorescence," Tissue Cell. 8:125-145; Johnson, A. R., and E. G. Erdos. 1977. "Metabolism of vasoactive peptides by human  
25 endothelial cells in culture: angiotensin I converting enzyme (kininase II) and angiotensinase," J. Clin. Invest. 59:684-695; and Patel, J. M., F. R.

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Yarid, E. R. Block, and M. K. Raizda. 1989. "Angiotensin receptors in pulmonary arterial and aortic endothelial cells," Am. J. Physiol. 256:C987-C993. Also, interruption of the endothelial autocrine angiotensin system, with either the angiotensin-converting enzyme inhibitor lisinopril or the angiotensin II receptor antagonist sar<sup>1</sup>, ile<sup>8</sup>-angiotensin II, has been shown to lead to increased endothelial cell migration and urokinase plasminogen activator (u-PA) activity. See Bell, L. and J. A. Madri. 1990. "Influence of the angiotensin system on endothelial and smooth muscle cell migration," Am. J. Pathol. 137:7-12. Although this work described a correlation between cell migration and u-PA activity, it did not establish a causal relationship between these biological functions.

In terms of clinical practice, restenosis following coronary angioplasty comprises a significant medical problem since it occurs within six months following 30-50% of the procedures performed and is associated with substantial patient morbidity and health care expenditures. All angioplasties cause removal of the endothelial cell lining of the blood vessel. The principal reasons for the restenosis are acute thrombus formation due to loss of the anti-thrombotic surface provided by the endothelial cells and neointima formation due to

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unchecked smooth muscle cell stimulation by blood-borne cells, including mononuclear cells, again due to the loss of the protective endothelial cell layer.

- 5           For example, Fishman, J. A., G. B. Ryan, M. J. Karnovsky. 1975. "Endothelial regeneration in the rat carotid artery and the significance of endothelial denudation in the pathogenesis of myointimal thickening," Laboratory Investigation. 32:339-351
- 10       show that loss of endothelial cells with denudation injury to the blood vessel wall is correlated with the subsequent formation of a neointima, or ingrowth of smooth muscle cells from the media into the intima and elaboration of increased amount of extracellular
- 15       matrix material resulting in a new intima. Schwartz, S. M., C. C. Haudenschild, and E. M. Eddy. 1978. "Endothelial regeneration: I. Quantitative analysis of initial stages of endothelial regeneration in rat aortic intima," Laboratory Investigation. 38:568-580
- 20       show that following denudation injury to an artery, in vivo, as would be expected following angioplasty or saphenous vein graft harvesting, remaining endothelial cells migrate to restore luminal integrity, and further Haudenschild, C. C. and S. M.
- 25       Schwartz. 1979. "Endothelial regeneration: II. Restitution of endothelial continuity," Laboratory Investigation. 41:407-418 show that injured vessel

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areas which are rapidly covered by a continuous layer of endothelium are protected from the development of neointima formation, or vessel lumen occlusion. Reidy, M. A. and S. M. Schwartz. 1981. "Endothelial regeneration: III. Time course of intimal changes after small defined injury to rat aortic endothelium," Laboratory Investigation. 44:301-308

5 also show that rapid coverage of the injured area is beneficial since removal of only a small number of endothelial cells from the vessel lumen allows rapid

10 recoverage of the area with endothelial cells and prevents the development of neointima formation, or vessel lumen occlusion. Further, Madri, J. A., M. A. Reidy, O. Kocher, and L. Bell. 1989. "Endothelial cell behavior following denudation injury is

15 modulated by TGF- $\beta$ 1 and fibronectin," Laboratory Investigation. 60:755-765 show that changes in in vivo endothelial cell migration correlate with in vitro endothelial cell migration assays. Hence,

20 rapid coverage of a denuded vessel segment, after angioplasty or following saphenous vein harvesting for bypass surgery for example, is an important parameter in preventing the vessel occlusion that commonly follows these procedures.

25 Occlusion of peripheral arterial and coronary artery bypass grafts is a further frequent and important clinical finding. Two-thirds of the

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saphenous vein coronary bypass grafts are either severely diseased or entirely occluded by six to eleven years following bypass surgery. Peripheral arterial bypass grafts have a similar fate. The occlusion is due to loss of endothelial cells from the surface of the vein graft during harvesting of the graft and at the time of initial surgery.

Synthetic grafts also exhibit high rates of occlusion. Initially, grafts of this type are not endothelialized. This results in a substantial incidence of early occlusion due to thrombosis. With time, the grafts become partially re-endothelialized by migration of arterial endothelial cells from the proximal and distal anastomotic sites or from ingrowth of capillary endothelial cells through the porous synthetic graft onto the luminal surface. However, the process of endothelial cell migration is normally slow and does not permit total coverage of the graft by arterial endothelial cells. Further, ingrowing capillary endothelial cells are less capable of inhibiting clot formation than arterial endothelial cells. Attempts to reseed peripheral grafts with autologous endothelial cells have demonstrated that incomplete coverage of the graft at the time of seeding results in graft closure and lack of clinical benefit of the seeding procedure.

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Thus, Zilla, P., R. Fasol, M. Deutsch, T. Fischlein, E. Minar, A. Hammerle, O. Krapicka, and M. Kadietz. 1987. "Endothelial cell seeding of polytetrafluoroethylene vascular grafts in humans: A preliminary report," Journal of Vascular Surgery. 6:535-541 and Fasol, R., P. Zilla, M. Deutsch, M. Grimm, T. Fischlein, and G. Laugfer. 1989. "Human endothelial cell seeding: Evaluation of its effectiveness by platelet parameters after one year," Journal of Vascular Surgery. 9:432-436 describe the absence of any significant improvement in platelet factors or function, platelet uptake on the graft surface, or distal blood flow up to one year after peripheral arterial bypass with a synthetic graft in patients who received synthetic grafts only partially coated with autologous endothelial cells. Ortenwall, P., H. Wadevik, J. Kutti, and B. Risberg. 1990. "Endothelial cell seeding reduces thrombogenicity of Dacron grafts in humans," Journal of Vascular Surgery. 11:403-410 did not observe any significant improvement in graft patency in patients who received synthetic graft partially coated with autologous endothelial cells. Thus reseed<sup>ing</sup> of synthetic grafts, or autologous grafts or denuded angioplasty sites, with endothelial cells will not result in clinical therapeutic benefit unless there is

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virtually complete coverage of the vessel segment with a continuous layer of endothelium.

Fibronectin is a ubiquitous glycoprotein found as a component of most extracellular tissues as well as in plasma. It serves as a principal component of many interstitial tissues and functions as an adhesion molecule that allows many cell types to adhere to the extracellular matrix. Fibronectin is also present in many embryonic tissues and allows for optimal cell migration and location during various cell migratory events that occur during development.

Fibronectin is known to be deposited at sites of injury and forms part of the provisional matrix at the injury site. For example, following balloon de-endothelialization, plasma fibronectin is deposited at the site of de-endothelialization and medial injury and fibronectin is also synthesized and deposited by reactive medial smooth muscle cells and endothelial cells.

In vitro studies have demonstrated that fibronectin elicits an enhancement of aortic smooth muscle cell migration and retards aortic endothelial cell migration compared to other matrix components. See Madri, J. A., Pratt, B.M., and Yanniarello-Brown, J., "Matrix-driven cell size changes modulate aortic endothelial cell proliferation and sheet migration", Am. J. Pathol., 132, 18, 1988; Pratt, B.M., Harris,

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- A.S., Morrow, J.S., and Madri, J.A., "Mechanisms of cytoskeletal regulation: modulation of aortic endothelial cell spectrin by the extracellular matrix", Am. J. Pathol., 117, 337, 1984; Leto, T.L.,
- 5 Pratt, B.M., and Madri, J.A., "Mechanisms of cytoskeletal regulation: modulation of aortic endothelial cell protein band 4.1 by the extracellular matrix", J. Cell Physiol., 127, 423, 1986; Madri, J.A., Pratt, B.M., and Yannariello-
- 10 Brown, J., "Endothelial cell-extracellular matrix interactions: matrix as a modulator of cell function", in Endothelial Cell Biology in Health and Disease, Simionescu, N. and Simionescu, M., Eds., Plenum Press, New York, 1988, 167; Pratt, B.M., Form,
- 15 D., and Madri, J.A., "Endothelial cell-extracellular matrix interactions", in Biology, Chemistry and Pathology of Collagen, Fleishmajer, R., Olsen, B. and Kuhn, K., Eds., Ann. N.Y. Acad. Sci., 460, 274, 1985; Madri, J.A., Kocher, O., Merwin, J.R., Bell, L., and
- 20 Yannariello-Brown, J., "The interactions of vascular cells with solid phase (matrix) and soluble factors", J. Cardiovasc. Pharmacol., 14, S70, 1989; and Merwin, J.R., Newman, W., Beall, D., Tucker, A., Madri, J.A., "Vascular cells respond differentially to
- 25 transforming growth factors beta<sub>1</sub> and beta<sub>2</sub>," Amer. J. Pathol., 138: 37-51, 1991. Further, using an in vivo model of balloon de-endothelialization, the

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chronically denuded injured segment has been shown to contain increased fibronectin on its luminal surface as well as throughout its neointimal compartment. See Madri, J.A., Reidy, M.A., Kocher, O., and Bell, L., "Endothelial cell behavior following denudation injury is modulated by TGF- $\beta$ 1 and fibronectin", Lab. Invest., 60, 755, 1989; and Basson, C.T., Kocher, O., Basson, M.D., Asis, A., and Madri, J.A., "Differential modulation of vascular cell integrin and extracellular matrix expression in vitro by TGF- $\beta$ 1 correlates with reciprocal effects on cell migration", J. Cell. Physiol., 153:118-128 1992. Reviews of these effects of fibronectin, as well as of other components of the extracellular matrix and of soluble factors associated therewith, can be found in Madri J.A., Bell L., "Vascular cell responses to injury: modulation by extracellular matrix and soluble factors," Cell Interactions in Atherosclerosis, Ed. by H. Robenek and N. Severs, CRC Press, Boca Raton, FL, Chapter 6, pp. 167-181, 1992; and Madri J.A., Bell L., Merwin J.R., "Modulation of vascular cell behavior by transforming growth factors  $\beta$ ," Molecular Reproduction and Development, 1992, 32:121-126.

In view of these effects on endothelial and smooth muscle cell migration, a reduction in the production of fibronectin by endothelial cells is

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desirable since such a reduction will enhance the migration of endothelial cells relative to smooth muscle cells. As discussed above, such enhanced migration means a reduced likelihood of restenosis at the site of, for example, a coronary angioplasty, as well as a reduced likelihood of occlusion of autologous and synthetic vascular grafts.

Mononuclear cells are a group of circulating blood cells comprising monocytes and T-lymphocytes. Monocytes are large, amoeboid, phagocytic leukocytes derived from bone marrow and containing one large nucleus. T-lymphocytes are smaller, ovoid cells, containing one nucleus and scant cytoplasm. T-lymphocytes are involved in the modulation of cellular and humoral immunity. In vivo, mononuclear cells have been found to adhere to vessel wall luminal cells at sites of atherosclerosis. See Stemme S., Holm J., Hansson G.K., "T lymphocytes in human atherosclerotic plaques are memory cells expressing CD45RO and integrin VLA-1. Arterioscl. and Thromb, 1992, 12: 206-211. The cells are believed to contribute to the development of this major disease process.

In vitro, mononuclear cells have been shown to adhere to migrating endothelial cells. See DiCorleto P.E., De La Motte C.A., "Characterization of the adhesion of the human monocytic cell line U937 to

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cultured endothelial cells," J. Clin. Invest., 1985, 75: 1153-1161. In terms of restenosis at the site of a vessel wall injury, e.g., an injury resulting from angioplasty, endarterectomy, or synthetic or autologous grafting, such adherence to endothelial cells can increase smooth muscle cell proliferation and migration as well as increasing extracellular matrix deposition, all of which can increase the likelihood of restenosis.

Genetic engineering of endothelial cells has been performed by a number of workers in the art. For example, Nabel, E. G., G. Plautz, F. M. Boyce, J. C. Stanley, and G. J. Nabel. 1989. "Recombinant Gene Expression in Vivo Within Endothelial Cells of the Arterial Wall," Science. 244:1342-1343, describe experiments in which a gene for the marker protein  $\beta$ -galactosidase was transferred to endothelial cells using a retroviral vector and the thus modified cells were seeded onto the walls of an artery in vivo using a double balloon catheter to isolate the section of the artery where the seeding took place. Nabel et al. report that up to four weeks after surgery, the seeded arteries were found to contain endothelial cells which expressed  $\beta$ -galactosidase.

Wilson, J. M., L. K. Birinyi, R. N. Salomon, P. Libby, A. D. Callow, and R. C. Mulligan. 1989.

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"Implantation of Vascular Grafts Lined with Genetically Modified Endothelial Cells," Science. 244:1344-1346, describe similar work wherein a  $\beta$ -galactosidase gene was transferred to endothelial cells using a retrovirus, the modified cells were seeded onto synthetic grafts, and the grafts were implanted in the carotid arteries of dogs. Five weeks later, the grafts were removed and found to still contain the genetically modified endothelial cells along their luminal surfaces.

Along these same lines, Dichek, D. A., R. F. Neville, J. A. Zwiebel, S. Freeman, M. B. Leon, and W. F. Anderson. 1989. "Seeding of Intravascular Stents with Genetically Engineered Endothelial Cells," Circulation. 80:1347-1353, describe the seeding of stainless steel stents with genetically engineered endothelial cells carrying in some cases a  $\beta$ -galactosidase gene and in others a human tissue-type plasminogen activator (TPA) gene. See also PCT Patent Publication No. WO 90/06997 (transfer of  $\beta$ -galactosidase, rat growth hormone, and human adenosine deaminase, CD-4, and TPA genes to endothelial cells and seeding of silicon coated polyurethane grafts and stainless steel stents with genetically engineered cells); and Zwiebel et al. 1989, supra.

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Direct in vivo transformation of arterial endothelial cells using retroviral particles or plasmid carrying liposomes is described in Nabel, E. G., G. Plautz, and G. J. Nabel. 1990. "Site-Specific Gene Expression in Vivo by Direct Gene Transfer into the Arterial Wall," Science. 249:1285-1288.  $\beta$ -galactosidase was again used as a marker protein, and evidence of transformation could be found 21 weeks after transfection.

The cellular src gene (c-src gene) was first identified in the late 1970's. See Stehelin, D., H. E. Varmus, J. M. Bishop, and P. K. Vogt. 1976. "DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA," Nature. 260:170-173; and Spector, D., H. E. Varmus, and J. M. Bishop. 1978a. "Nucleotide sequences related to the transforming gene of avian sarcoma virus are present in DNA of uninfected vertebrates," Proc. Nat. Acad. Sci. USA. 75:4102-4106. The gene appears to be present in all animal species and is highly conserved. It encodes a 60,000 dalton protein, tyrosine kinase, which is localized on the cytoplasmic side of the plasma membrane. The c-src protein will be designated herein as pp60<sup>c-src</sup>.

pp60<sup>c-src</sup> is a representative molecule of the src-family of membrane-bound tyrosine kinases including, but not limited to yes, lck, and fyn. (See

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C. A. Koch, D. Anderson, M. F. Moran, C. Ellis, and T. Pawson. 1991 "SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins," Science. 252:668-674.) Certain critical and highly conserved noncatalytic domains in the src family of tyrosine kinases are called Src homology (SH) regions 2 and 3 and are involved in protein-protein interactions. These Src-homology domains are also found in a series of critical molecules, including, but not limited to fyn, lck, yes, PLC, p85, tensin, crk, vav, GAP, fps, arg, dabl, hck, blk, fgr, and nck. These domains are believed to regulate various cell effects of src and related molecules including, but not limited to, signal transduction pathways of tyrosine kinase receptors.

The sequence of the c-src gene has been known for some time. See Takeya, T. and H. Hanafusa. 1983. "Structure and Sequence of the Cellular Gene Homologous to the RSV src Gene and the Mechanism for Generating the Transforming Virus," Cell. 32:881-890. A copy of the nucleotide sequence for the coding region of this gene in the chicken and of the resulting pp60<sup>c-src</sup> protein as published by Takeya and Hanafusa appear as SEQ. ID. NOS. 1 and 2, respectively, set forth below. The corresponding human sequences are set forth as SEQ. ID. NOS. 3 and 4. See Anderson, S. K., C. P. Gibbs, A. Tanaka, H.

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- Kung, and D. Fujita. 1985. "Human Cellular src Gene: Nucleotide Sequence and Derived Amino Acid Sequence of the Region Coding for the Carboxy-Terminal Two-Thirds of pp60<sup>c-src</sup>," Molecular and Cellular Biology. 5:1122-1129 and Tanaka, A., C. P. Gibbs, R. R. Arthur, S. K. Anderson, H. Kung, and D. Fujita. 1987. "DNA Sequence Encoding the Amino-Terminal Region of the Human c-src Protein: Implications of Sequence Divergence among src-Type Kinase Oncogenes," Molecular and Cellular Biology. 7:1978-1983.

Various functions and properties of the c-src gene have been described in the literature. For example, Shalloway, D., P. M. Coussens, and P. Yaciuk. 1984. "Overexpression of the C-src Protein Does Not Induce Transformation of NIH 3T3 Cells," Proc. Natl. Acad. Sci. USA. 81:7071-7075, have shown that genetically engineered mouse NIH 3T3 fibroblast cells which overexpress pp60<sup>c-src</sup> are not malignant. Azarnia, R. S. Reddy, T. E. Kmiecik, D. Shalloway, and W. R. Loewenstein. 1988. "The Cellular src Gene Product Regulates Junctional Cell-to-Cell Communication," Science. 23:398-401, have shown that overexpression of pp60<sup>c-src</sup> in NIH 3T3 cells causes a reduction in cell-to-cell transmission of molecules in the 400 to 700 dalton range. See also Loewenstein, W. R., and R. Azarnia. 1988. "Regulation of Intercellular Communication and Growth by the

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Cellular src Gene," Annals New York Academy of Sciences. 551:337-346. Soriano, P., C. Montgomery, R. Geske, and A. Bradley. 1991. "Targeted Disruption of the c-src Proto-Oncogene Leads to Osteopetrosis in Mice," Cell. 64:693-702, have shown that mutation of the c-src gene results in a marked decrease in the rate of bone resorption in mice, i.e., osteopetrosis, thus suggesting that the normal c-src gene plays a role in bone formation.

10 In addition to the foregoing, Warren, S. L., L. M. Handel and W. J. Nelson. 1988. "Elevated expression of pp60<sup>c-src</sup> alters a selective morphogenetic property of epithelial cells in vitro without a mitogenic effect," Mol. Cell. Biol. 8:632-646, have shown that the overexpression of pp60<sup>c-src</sup> in Madin-Darby canine kidney cells causes those cells to undergo changes in shape, including the formation of elongated cell processes having lengths in the range of 100 to 200 microns.

20 A gene related to the c-src gene is the oncogene v-src which forms part of the genome of the Rous sarcoma virus and causes that virus to produce sarcomas in chickens. v-src phosphorylates many more substrates compared to c-src and overexpression of v-src elicits a transformed phenotype while overexpression of c-src does not. See Takeya and Hanafusa, supra; and Hunter, T. 1987. "A Tail of Two

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src's: Mutatis Mutandis," Cell. 49:1-4. As with many malignant cells, cells infected with the Rous sarcoma virus have been found to exhibit increased production of urokinase-type plasminogen activator (u-PA). In particular, Bell, S. M., R. W. Brackenbury, N. D. Leslie and J. L. Degen. 1990. "Plasminogen activator gene expression is induced by the src oncogene product and tumor promoters," J. Biol. Chem. 265:1333-1338, have correlated the increased production of u-PA after transformation of chicken embryo fibroblasts by the Rous sarcoma virus with an increase in cellular u-PA mRNA.

Significantly, none of the prior art in any way discloses or suggests the surprising results achieved by the present invention wherein increased expression of pp60<sup>c-src</sup> by genetically engineered endothelial cells has been found to result in 1) enhanced migration of the cells, i.e., an enhanced ability to repair the endothelial lining of damaged vessels and/or an enhanced ability to form an endothelial lining on grafts or stents; 2) enhanced urokinase-type plasminogen activator activity, i.e., an enhanced ability to dissolve or prevent the formation of the thrombi normally associated with vascular surgical procedures; 3) reduced mononuclear cell (e.g., monocyte) adhesion to the cells, e.g., less likelihood of the formation of atherosclerotic

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plaques in the region of the genetically engineered cells; and 4) reduced fibronectin production by the cells, e.g., reduced migration of smooth muscle cells in the region of the cells.

5     SUMMARY OF THE INVENTION

          In view of the foregoing, it is an object of this invention to provide genetically engineered endothelial cells having improved therapeutic properties. More particularly, it is an object of  
10     the invention to provide genetically engineered endothelial cells which migrate at higher rates than corresponding endothelial cells which have not been genetically engineered. It is also an object of the invention to provide genetically engineered  
15     endothelial cells which have an enhanced ability to inhibit the formation of thrombi and/or to dissolve thrombi once they have formed. It is a further object of the invention to provide genetically engineered endothelial cells to which mononuclear  
20     cells, e.g., monocytes, are less likely to adhere than endothelial cells which have not be genetically engineered. It is an additional object of the invention to provide genetically engineered endothelial cells which produce reduced amounts of  
25     fibronectin.

          With regard to clinical applications, it is an object of the invention to provide genetically

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engineered endothelial cells which can be used to improve the success of such surgical procedures as coronary angioplasty and vessel graft and stent implantation.

5 To achieve the foregoing and other objects, the invention provides endothelial cells which have been genetically engineered to produce increased amounts of pp60<sup>c-src</sup>. As shown in the examples presented below, such genetically engineered cells exhibit  
10 enhanced cell migration, enhanced u-PA production, reduced mononuclear cell adhesion, and reduced fibronectin production and thus address the long-standing problems in the field of vascular surgery of endothelial layer reconstruction and  
15 restenosis inhibition, including thrombus inhibition, at the surgical site.

The accompanying figures, which are incorporated in and constitute part of the specification, illustrate certain aspects of the invention, and  
20 together with the description, serve to explain the principles of the invention. It is to be understood, of course, that both the figures and the description are explanatory only and are not restrictive of the invention.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the structure of the three retroviral vectors used in the examples. The abbreviations used in this figure are as follows:

5 rectangular box with a central bar -- LTR; open triangle -- 5' splice site; open circle --  $\psi$  packaging signal; closed triangle -- 3' splice site; solid arrow --  $\text{Neo}^r$ ; dotted arrow --  $\Delta i295$  (kinase-deficient) mutant; stripped arrow -- c-src

10 gene; double vertical bar and attached dotted line -- SV40 early region promoter-enhancer; solid line -- rat genomic DNA; E -- EcoRI; B -- BamHI.

Figure 2 shows the src kinase activity as determined by the in vitro kinase assay (see below)

15 for cells infected with the vectors of Figure 1. The following abbreviations are used in this figure and in Figures 3, 4, 6, and 8: endothelial cells expressing elevated levels of c-src -- C-SRC; endothelial cells expressing the kinase negative

20 mutant c-src, met-295 -- met-295; endothelial cells expressing Tn5 aminoglycoside phosphotransferase alone --  $\text{Neo}^r$ .

Figure 3 shows migration rates relative to that of unmodified control cells ("BAEC CONT") of the

25 C-SRC, met-295, and  $\text{Neo}^r$  cells. The "\*" indicates  $P < 0.001$  for the C-SRC cells vs. noninfected endothelial cells.

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Figure 4 shows u-PA activity during migration of the C-SRC, met-295, and Neo<sup>r</sup> cells relative to that of unmodified control cells. u-PA activity was  $4.9 \pm 0.05$  mPU/ $\mu$ g cell protein in the control cells. The "\*" indicates  $P < 0.001$  for the C-SRC cells vs. noninfected endothelial cells. Similar results (not shown) were obtained with standard plasminogen zymography.

Figures 5A and 5B are photomicrographs at the leading edge of migration of Neo<sup>r</sup> cells (Figure 5A) and C-SRC cells (Figure 5B) which have been stained for u-PA. The bar represents 50  $\mu$ m.

Figure 6 shows the effect of antisera to bovine u-PA on c-src induced endothelial cell migration. The "\*" indicates  $P < 0.001$  for the C-SRC cells vs. Neo<sup>r</sup> cells, and the "+" indicates  $P < 0.01$  for the C-SRC cells incubated with antisera vs. untreated cells.

Figure 7 illustrates the effect of overexpression of pp60<sup>c-src</sup> on the adhesion of monocytes, specifically, U937 cells, to bovine aortic endothelial cells (BAEC) at the fronts of migrating monolayers. Each bar group represents the number of U937 cells bound in an area of  $0.01 \text{ mm}^2$ , starting from the edge of the migrating front (1) and moving toward the center of the dish (2,3,4). Open boxes = Numbers of U937 cells bound to control migrating

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BAEC. Shaded boxes = Numbers of U937 cells bound to cultures of migrating BAEC that stably expressed the neomycin resistance gene (Neo<sup>r</sup>). Black boxes = Numbers of U937 cells bound to cultures of migrating BAEC that stably express elevated levels of pp60<sup>c-src</sup>.

Figure 8 shows fibronectin protein levels associated with 3 day migrating bovine aortic endothelial cells stably expressing the neomycin resistance gene (Neo<sup>r</sup>) or the neomycin resistance gene and the c-src gene determined by the ELISA technique described below. Overexpression of pp60<sup>c-src</sup> is associated with decreased endothelial cell fibronectin protein at day 3 compared to BAEC expressing only the neomycin resistance gene.

Figure 9 illustrates the effect of TGF- $\beta$ 1 treatment on the adhesion of U937 cells to the fronts of migrating monolayers of BAEC overexpressing pp60<sup>c-src</sup>. Each bar group represents the number of U937 cells bound in an area of 0.01 mm<sup>2</sup>, starting from the edge of the migrating front (1) and moving toward the center of the dish (2,3,4). Open boxes = Numbers of U937 cells bound to control c-src transfected migrating BAEC. Shaded boxes = Numbers of U937 cells bound to cultures of c-src transfected migrating BAEC that have been treated with 0.5 ng/ml TGF- $\beta$ 1.

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DESCRIPTION OF THE PREFERRED EMBODIMENTS

As discussed above, the present invention relates to endothelial cells which have been genetically engineered to express elevated levels of  
5 the pp60<sup>c-src</sup> protein.

The endothelial cells are obtained from the lining of a portion of the vascular system, e.g., a blood vessel or capillary, and are maintained in a tissue culture or other suitable biological medium.  
10 The cells will generally be from the patient being treated, although they can be from another individual or another species, e.g., porcine or bovine endothelial cells which can be readily obtained in large quantities, provided that anti-rejection  
15 therapies are used to control rejection of the non-autologous cells upon implantation. See, for example, copending U.S. patent application Serial No. 07/906,394, filed June 29, 1992, and entitled "Universal Donor Cells."

20 Insertion of one or more copies of the coding sequence of the c-src gene into the endothelial cells is accomplished using conventional recombinant genetic engineering techniques for transforming cells now known or subsequently developed. For example,  
25 retroviral vectors, electroporation, calcium-phosphate techniques, adenovirus vectors, or other means of gene transfer can be used for this purpose.

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Whatever technique is chosen, a heterologous promoter will be included with the structural gene so that the pp60<sup>c-src</sup> protein or a selected portion thereof (see below) will be expressed in the modified endothelial cell.

Various vectors containing the coding sequence of the c-src gene are known in the art. For example, copies of this gene have been previously cloned into the pMc-srcΔiSVneo and p5H plasmids. See Warren et al., supra, and Levy, J. B., H. Iba, and H. Hanafusa. 1986. "Activation of the transforming potential of pp60<sup>c-src</sup> by a single amino acid change," Proc. Natl. Acad. Sci. USA 83:4228-4232. Deposits of the related v-src gene are available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20852, United States of America, in the vectors pEcoRIB, Rous sarcoma virus v-src oncogene (v-src), and pPvuIIE, Rous sarcoma virus v-src oncogene (v-src), (ATCC Accession Nos. 41005 and 41006, respectively). These vectors were cloned from Rous sarcoma virus Schmidt Rupp A2 and are available from the ATCC in freeze-dried Escherichia coli HB101.

In view of the fact that c-src gene is highly conserved in all species and the fact that the c-src and v-src genes share substantial regions of homology (see Takeya and Hanafusa, supra), these previously

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cloned c-src genes and/or the deposited v-src genes can be readily used to screen genetic libraries for the c-src gene for any particular species that may be desired. Examples of the types of approaches which can be used appear in the work of Anderson et al., 1985, supra, and Tanaka et al., 1987, supra, which are directed to the human c-src gene and protein. For clinical applications, the human c-src gene, sequenced by these workers, is generally preferred.

The DNA coding and amino acid sequences for representative c-src genes are set forth below in the Sequence Listing. In particular, SEQ. ID. NOS. 1 and 2 set forth the nucleotide and amino acid sequences for the c-src gene and the pp60<sup>c-src</sup> protein in the chicken, while SEQ. ID. NOS. 3 and 4 set forth the corresponding sequences in the human.

As detailed by Anderson et al., 1985, supra, and Tanaka et al., 1987, supra, these nucleotide and amino acid sequences exhibit very high levels of homology. Thus, the average amino acid sequence homology for these two very diverse species is 98% for exons 3 through 12. Significantly, the kinase active region, as well as the SH2 and SH3 regions which affect protein-protein interactions (see Koch et al., 1991, supra), are contained in these highly conserved portions of the protein molecule. The average homology for exon 2 is 71%, which is still

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high, although not as high as for exons 3 through 12. As noted by Anderson et al., 1985, supra, exon 1 codes for a 5' untranslated region of mRNA and thus does not appear in the pp60<sup>c-src</sup> protein.

5           Similar homologies are seen on the DNA level. Thus, for exons 2-12, the percentage of identical nucleotides in the coding sequences of the chicken c-src gene and the human c-src gene is 85.4% (i.e., 1,374 nucleotides out of a total of 1,608). See  
10 Tanaka et al., 1987, supra, Table 1. Moreover, as evidenced by the fact that 94.2% of the amino acids for the chicken and human proteins are identical, Id., the majority of nucleotide changes are silent, third-position codon changes resulting in no amino  
15 acid substitutions.

As recognized in the art, the DNA coding and amino acid sequences of the c-src gene/protein for other species are similarly conserved.

In view of these homologies, the terms "c-src  
20 gene" and "pp60<sup>c-src</sup> protein" are used herein to describe these families of substantially similar DNA sequences and resulting proteins, it being understood that any particular member of the family can be used for any particular application either in identical  
25 form or with modification provided such modifications do not prevent the gene/protein from exhibiting the effects of enhanced cell migration, enhanced u-PA

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activity, reduced mononuclear cell, e.g., monocyte, adhesion, and/or reduced fibronectin production. For example, it has been found that the migration and u-PA enhancements described in the examples presented below can also be achieved for a c-src gene which codes for asp, rather than gly, at amino acid position 63 in SEQ. ID. NO. 1.

In this regard, as discussed above, various subregions of the c-src gene/protein are highly conserved, including the SH2 and SH3 domains and the kinase active portion of the protein molecule. These regions, individually or in combination, as well as other subregions of the c-src gene, can be used in the practice of the invention provided they produce the desired effects of enhanced endothelial cell migration, enhanced u-PA activity, reduced mononuclear, e.g., monocyte, adhesion, and/or reduced fibronectin production. Depending upon their length, such subregions can be obtained by direct synthesis, by digesting the c-src gene with restriction enzymes, by polymerase chain reaction amplification of all or a part of the desired sequence, or by combinations of such techniques.

The transformation of the endothelial cells is preferably performed in vitro with the transformed cells being implanted directly in the vessel wall using techniques of the type described in Nabel et

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al., 1989, supra, or used to coat a graft, stent, or similar device which is then implanted. See Wilson et al., 1989, supra, Dichek et al., 1989, supra, Zwiebel et al., 1989, supra, and PCT Patent  
5 Publication No. WO 90/06997.

More particularly, endothelial cells, which have been genetically modified to express elevated levels of pp60<sup>c-src</sup>, can be implanted clinically in a patient's coronary artery by:

- 10 1. Harvesting the patient's endothelial cells or selecting endothelial cells which can be implanted in the patient through the use of, for example, anti-rejection techniques or processes.
- 15 2. Inserting the c-src gene or a part thereof into the endothelial cells using, for example, a retroviral vector and, in particular, a retroviral packaging system which produces viral vector particles which are free of replicating virus. See, for example, Varmus, H. E. 1982. "Form and Function of Retroviral Proviruses,"  
20 Science. 216:812-820; and Mann, R., R. C. Mulligan, and D. Baltimore. 1983. "Construction of a Retrovirus Packaging Mutant and Its Use to Produce Helper-Free Defective Retrovirus," Cell.  
25 33:153-159.
3. Performing diagnostic catheterization of the patient to determine the severity, location and

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amenability of the coronary (or peripheral) artery disease to angioplasty, atherectomy, laser therapy, or other forms of mechanical revascularization.

- 5      4. Assuming step (3) determines that therapeutic angioplasty is appropriate, performing a standard balloon angioplasty procedure.
- 10      5. Using a standard wire exchange technique, removing the balloon angioplasty catheter and replacing it with a double balloon catheter having an infusion exit port positioned between the two balloons.
- 15      6. Positioning the double balloon catheter tip in the angioplastied coronary artery with the double balloons straddling the denuded segment of the artery, i.e., the portion of the artery in which the endothelial lining has been removed by the angioplasty procedure.
- 20      7. Gently inflating the double balloons while supporting the distal coronary circulation with standard perfusion techniques.
- 25      8. Introducing the c-src modified endothelial cells into the extracorporeal end of the double balloon catheter and infusing the cells into the isolated space in the blood vessel between the two balloons at a concentration of, for example,

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2-10 x 10<sup>6</sup> cells per 10 milliliters of solution to seed the denuded portion of the vessel.

9. After approximately twenty to thirty minutes, deflating the double balloon catheter so as to restore normal antegrade coronary perfusion.
10. Removing the double balloon catheter followed by standard post catheterization procedures.

Similarly, a synthetic or autologous vascular graft or stent can be coated with the c-src modified endothelial cells and then implanted in a patient by:

1. Preparing c-src modified endothelial cells as described in steps (1) and (2) above.
2. Performing diagnostic catheterization of the patient to determine the severity, location and amenability of the coronary (or peripheral) artery disease to vascular bypass surgery with autologous, synthetic, or other graft material.
3. In the case of a synthetic graft or stent, coating the graft or stent with Type I collagen and fibronectin in saturating amounts greater than or equal to 25 µg/ml in carbonate buffer, pH 9.4; in the case of an autologous graft, harvesting the saphenous vein or other vessel using conventional surgical techniques.
4. Cannulating the proximal end and ligating the distal end of the synthetic or saphenous vein graft.

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5. Injecting the c-src modified endothelial cells, at a concentration of, for example,  $2-10 \times 10^6$  cells per 10 milliliters of solution, through the proximal cannulation port into the lumen of the graft and rotating the graft for approximately 60 minutes to allow the c-src modified endothelial cells to cover the graft surface.
6. Implanting the seeded graft in the coronary or peripheral artery using standard fine surgical techniques.

In either case, because the genetically modified endothelial cells express elevated levels of pp60<sup>c-src</sup>, they exhibit reduced fibronectin production and, at least partially as a result thereof, enhanced cell migration thus providing improved and rapid coverage of the denuded vessel or graft in the case of angioplasty or bypass surgery, respectively. Moreover, because the cells also exhibit enhanced u-PA activity, the probability of thrombosis at the vessel wall surface is reduced. Further, because the cells exhibit reduced mononuclear cell adhesion, the likelihood of restenosis of the vessel segment as well as the likelihood of plaque formation are reduced.

Without intending to limit it in any manner, the present invention will be more fully described by the

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following examples. The materials and methods which are common to the examples are as follows.

I. Cell culture, migration, proliferation and size.

Bovine calf aortic endothelial cells (BAEC) were  
5 isolated, cultured with DME (Gibco Laboratories,  
Grand Island, NY), and characterized as described in  
Bell, L. and J. A. Madri. 1989. "Effect of platelet  
factors on migration of cultured bovine aortic  
endothelial and smooth muscle cells," Circ. Res.  
10 65:1057-1065. See also Madri J.A., Dreyer B.,  
Pitlick F., Furthmayr H., "The collagenous components  
of subendothelium: Correlation of structure and  
function," Lab. Invest., 1980, 43: 303-315. For the  
monocyte adhesion and fibronectin production  
15 experiments, the cells were cultured in DMEM (Gibco)  
with 10% fibronectin-free fetal calf serum (Gibco).

Endothelial cells were first seeded into the  
middle of a steel fence and allowed to attach to the  
underlying Type I collagen matrix below; after cell  
20 attachment, the fence was removed and, with the loss  
of contact inhibition, the monolayer of cells  
commenced radial migration outward over a 6 day  
period. For the monocyte adhesion and fibronectin  
production experiments, a laminin matrix, as opposed  
25 to a Type I collagen matrix, was used and the  
monolayer of cells commenced radial migration outward  
over a 3 day period, as opposed to a 6 day period.

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The laminin matrix was chosen because BAEC migrate well on laminin (see Madri J.A., Pratt B.M., Yannariello-Brown J., "Matrix driven cell size changes modulate aortic endothelial cell proliferation and sheet migration," Amer. J. Pathol. 1988, 132:18-27) and U937 cells do not exhibit detectable adhesion to this material. In this way, U937 cell adhesion to the baseline substratum was not a complicating factor in the endothelial/monocyte adhesion studies.

The role of u-PA in mediating changes in cell migration was evaluated during 3 day migrations of endothelial cells treated with either 5% immune anti-bovine urokinase antiserum administered daily or 5% nonimmune rabbit serum administered daily. See Saksela, O., and D. B. Rifkin. 1990. "Release of basic fibroblast growth factor-heparan sulfate complexes from endothelial cells by plasminogen activator-mediated proteolytic activity," J. Cell Biol. 110:767-775.

The possible contribution of changes in cell proliferation during migration was measured by trypsinizing migrating cells and counting aliquots in a Coulter counter (Coulter Electronics, Inc., Hialeah, FL).

Cell sizes during migration were measured on approximately 100 cells per treatment by morphometric

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analysis with a digitizing tablet. See Bell and Madri, 1990, supra.

## II. Immunoblot analysis.

Cell protein was extracted with RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2, 1 mM PMSF, and 0.2 mM vanadate) and normalized for total protein using the bicinchonic acid assay. See Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson and D. C. Klenk. 1985. "Measurement of protein using bicinchonic acid," Anal. Biochem. 150:76-85.

Equal protein loads of the cell lysates were run on a 6% reducing polyacrylamide gel, transferred to nitrocellulose paper, blocked with 4% PBSA, and incubated with anti-src monoclonal antibody (Mab327) (Oncogene Science, Inc. Manhasset, NY). See Knecht, D. A. and R. L. Dimond. 1984. "Visualization of antigenic proteins on Western blots," Anal. Biochem. 136:180-184; and Blake, M. S., K. H. Johnston, G. J. Russell-Jones and E. C. Gotschlich. 1984. "A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots," Anal. Biochem. 136: 175-179. This antibody also immunoblots and precipitates pp60<sup>c-src</sup> from cells derived from a wide variety of species including bovine vascular smooth muscle cells and

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chicken, rat, and canine cells. See DiSalvo, J., D. Gifford, and A. Kokkinakis. 1988. "pp60<sup>c-src</sup> kinase activity in bovine coronary extracts is stimulated by ATP," Biochem. Biophys. Res. Commun. 153:388-394;

5 Dorai, T., and L. H. Wang. 1990. "An alternative non-tyrosine protein kinase product of the c-src gene in chicken skeletal muscle," Mol. Cell. Biol. 10:4068-4079; and Warren et al., 1988, supra. Normal mouse sera was found not to immunoblot or precipitate

10 the appropriate 60-kD moiety from bovine, canine, or rat cells.

The immunoblots were developed with rabbit anti-mouse IgG and <sup>125</sup>I-protein A and then exposed to XAR film (Eastman Kodak Co., Rochester, NY) at -70°C.

15 Quantitative determinations of relative amounts of the src protein were performed using a densitometer (Hoefer Scientific Instruments, San Francisco, CA).

### III. Kinase assay.

Kinase activity was determined using an assay

20 which relies on the ability of c-src to autophosphorylate. That is, c-src has the ability to transfer a phosphate group to itself from a phosphate donor such as ATP. Accordingly, by labeling ATP in the gamma position with a p<sup>32</sup> isotope, the kinase

25 activity of c-src can be determined by quantifying the amount of p<sup>32</sup> incorporated into the c-src protein.

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In accordance with this procedure, cell protein was extracted with RIPA buffer and normalized for total protein as above. Equal amounts of cell protein were then precleared with normal mouse IgG and incubated overnight with MAb327. The antigen/antibody complexes containing active enzyme were precipitated with Protein A sepharose beads, washed with RIPA buffer and subsequently suspended in a substrate-containing buffer composed of 20 mM Tris-HCl, pH 7.2, 5 mM MgCl<sub>2</sub> with 10  $\mu$ Ci  $\gamma$ <sup>32</sup>ATP/reaction for 10 minutes at 30°C. See Warren et al., 1988, supra. The reaction was stopped with excess unlabeled ATP. The beads were boiled in solubilization buffer, loaded on a 10% reducing polyacrylamide gel, and the gel was developed with Kodak XAR film at -70°C. Quantitative determinations of relative amounts of c-src kinase activity were performed using a Hoefer densitometer to measure the levels of incorporation of p<sup>32</sup> into the c-src protein.

#### IV. Plasminogen activator activity assay.

Urokinase plasminogen activator activity was measured using the chromogenic substrate H-D-norleucyl-hexahydrotyrosyl-lysine-p-nitroanilide diacetate salt (American Diagnostica, Inc., Greenwich, CT) at a final concentration of 250 mM and human plasminogen at a final concentration of

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25  $\mu\text{g/ml}$  in 120 mM Tris-HCl pH 8.7 as described in Bell and Madri, 1990, supra. The results of this assay were confirmed by plasminogen zymography, modified from Granelli-Piperino and Reich, with final concentrations of non-fat milk 4%, 0.1 M Tris-HCl pH 7.2, 8  $\mu\text{g/ml}$  plasminogen, and 1.25% agar. See Granelli-Piperino, A. and E. Reich. 1978. "A study of proteases and protease-inhibitor complexes in biological fluids," J. Exp. Med. 148:223-234.

10 V. Northern blot analysis.

Total cellular RNA was extracted with 4M guanidinium HCl, 5 mM sodium citrate pH 7.0, 0.1%  $\beta$ -mercaptoethanol, and 0.5% Sarkosyl, centrifuged on a cushion of 5.7 M CsCl in 0.1 M EDTA, and re-extracted with a 4:1 mixture of chloroform and 1-butanol and ethanol precipitation. See Maniatis, T., E.F. Fritsch and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 196. Total cellular RNA, 20  $\mu\text{g}$  per lane, was electrophoresed through a 1% formaldehyde gel, transferred to Nytran filters and prehybridized with 0.5 M sodium phosphate pH 7.2, 7% SDS, 1% BSA, 1 mM EDTA for 2 hours at 65°C. See Mahmoudi, M. and V.K. Lin. 1989. "Comparison of two different hybridization systems in Northern transfer analysis," Biotech 7:331-333. The blot was hybridized overnight at 65°C with a labeled

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dCT<sup>32</sup>Pc-src DNA probe, washed twice with 2 x SSC and 0.1% SDS for 30 minutes at 65°C, and developed against Kodak XAR film at -70°C. See Warren et al., 1988, supra. Quantitative determinations of relative amounts of the src mRNA were performed using a Hoefer densitometer. All immunoblots, kinase assays, and RNA hybridizations were performed at least two times.

#### VI. Transfections and infections.

The c-src coding sequence (SEQ. ID. NO. 1) was spliced into the helper-free Moloney retroviral vector MsrcΔiSVneo(+). Id. MsrcΔi295SVneo(+), a similar virus which encodes a kinase negative mutant of c-src, Met-295, and a control virus, Hippo42, that encodes Tn5 aminoglycoside phosphotransferase (Neo<sup>r</sup>) were also employed. See Jove, R., S. Kornbluth and H. Hanafusa. 1987. "Enzymatically inactive p60c-src mutant with altered ATP-binding site is fully phosphorylated in its carboxy-terminal regulatory region," Cell. 50:937-43. See also Bell L., Luthringer D.J., Madri J.A., Warren S.L., "Autocrine angiotensin system regulation of endothelial cell behavior involves modulation of pp60<sup>c-src</sup> expression," J. Clin. Invest. 1992, 89: 315-320. ψ-2 cells were transfected with plasmid DNA using polybrene/DMSO shock. ψ-2 cells were selected in G418 (Sigma Chemical Co.) and the filtered media from resistant cells was used to infect ψ-AM cells as

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described in Cone, R.D. and R.C. Mulligan. 1984.  
"High-efficiency gene transfer into mammalian cells:  
generation of helper-free recombinant retrovirus with  
broad mammalian host range," Proc. Natl. Acad. Sci.  
5 81:6349-6353. The resulting amphotropic virus was  
then used to infect subconfluent BAEC which were  
selected in G418, 400  $\mu\text{g/ml}$ , until all uninfected  
BAEC were killed (10 days). For the monocyte  
adhesion and fibronectin production experiments, the  
10 BAEC were selected in G418 for 3 weeks. Also, for  
these experiments, the polybrene/DMSO shock treatment  
was conducted for 4 minutes at 20% DMSO and 30  $\mu\text{g/ml}$   
polybrene, the selection of the transfected  $\psi$ -2 cells  
was performed using a G418 concentration of  
15 400  $\mu\text{g/ml}$ , the filtering of the media containing  
ecotropic viral particles was performed using 0.45  
micron membranes, and the  $\psi$ -AM were infected at a  
polybrene concentration of 8  $\mu\text{g/ml}$ .

Infection of the endothelial cells and transfer  
20 of the c-src gene was readily accomplished following  
these techniques. The transformed cells were found  
to grow stably for at least six months and to  
maintain their genetically altered properties for at  
least 2 years when frozen.

## 25 VII. Immunofluorescence.

Migrating cells were washed four times with PBS,  
fixed with periodate-lysine-paraformaldehyde.

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fixative, permeabilized with 0.2% Triton X-100, and blocked overnight with PBS with 3% BSA. Cells were incubated with either nonimmune rabbit serum or rabbit anti-bovine u-PA antisera and rhodamine  
5 conjugated goat anti-rabbit secondary antibody. Cells were examined on a MRC-600 confocal microscope (Bio-Rad Laboratories, Richmond, CA). Cells incubated with nonimmune serum demonstrated no detectable staining.

10 VIII. Monocytic Cell Adhesion Assay

U937 cells obtained from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20852, United States of America, were used in the adhesion assay (accession number ATCC CRL  
15 1593). These cells are human histiocytic lymphoma cells obtained from the pleural effusion of a 37-year-old Caucasian male with diffuse histiocytic lymphoma (Sundstrom and Nilsson, Int. J. Cancer 17:565-577, 1976). The cells were propagated in the  
20 medium suggested by the ATCC, i.e., RPMI 1640, 90%, fetal bovine serum, 10%. U937 cells were chosen for these experiments since they are widely used as a representative of monocyte cells.

Adhesion of the U937 cells to BAEC cultures that  
25 had been migrating for three days was assessed in the assay. BAEC cultures that had been migrating for this period of time were washed with RPMI 1640

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medium, incubated with  $3 \times 10^6$  U937 cells in 1.3 ml RPMI 1640 for two hours at 37°C, fixed in 10% buffered formalin/2% glutaraldehyde, washed and stained with hematoxylin. After the fixation and staining procedure, bound U937 cells were counted (nine adjacent microscopic fields, starting from the migrating edge and progressing inward, toward the center of the dish; each field representing an area of  $0.10 \text{ mm}^2$ ). Data are expressed as adherent U937 cells/field. Shown are mean values and standard errors. Significances were obtained using the unpaired t-test.

#### IX. ELISA protein determinations.

Purified human plasma fibronectin and affinity-purified antibodies against fibronectin were isolated and purified as described in Basson C.T., Knowles W.J., Abelda S., Bell L., Castronovo V., Liotta L.A., Madri J.A., "Spatiotemporal segregation of endothelial cell integrin and non-integrin extracellular matrix binding proteins during adhesion events," J. Cell. Biol. 1990, 110: 789-802, and Madri J.A., Roll F.J., Furthmayr H., Foidart J.M., "The ultrastructural localization of fibronectin and laminin in the basement membranes of the murine kidney," J. Cell. Biol. 1980, 86: 682-687. The procedures of these references were also used to isolate and purify laminin from EHS tumor.

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Concentrations of fibronectin in the medium and cell layers of migrating endothelial cells were determined by a quantitative ELISA inhibition assay sensitive to 0.1 ng. See Madri J.A., Pratt B.M., Tucker A.M., "Phenotypic modulation of endothelial cells by transforming growth factor- $\beta$  depends upon the composition and organization of the extracellular matrix," J. Cell. Biol. 1988, 106:1375-1384.

X. Statistical analysis.

Changes in migration, proliferation, and u-PA activity were analyzed by analysis of variance and correction was made for multiple comparisons using the method of Bonferroni. Statistical significance was assumed for  $P < 0.05$ . For the monocyte adhesion and fibronectin production experiments, statistical analyses were performed using the STATWORKS program and a MACINTOSH IIci computer.

Example 1

Enhanced Migration of Genetically

Engineered Endothelial Cells

This example demonstrates the enhanced migration of endothelial cells which have been genetically engineered to express higher than normal levels of pp60<sup>c-src</sup>.

Using the amphotropic, helper-free retroviral vector Msrc $\Delta$ iSVneo(+) described above, the c-src gene was transferred into subconfluent bovine aortic

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endothelial cells (BAEC). In addition, BAEC were infected with the kinase negative c-src mutant, Mcsrc $\Delta$ i295SVneo(+), and with a control virus, Hippo42, that encodes Tn5 aminoglycoside phosphotransferase (Neo<sup>r</sup>). The structures of the retroviral vectors used for these transformations are shown in Figure 1.

With regard to their general behavior, the c-src infected endothelial cells did not overgrow monolayers or proliferate in suspension, were contact inhibited, exhibited sheet migration, and retained Factor VIII staining. The proliferation rates (determined by cell counting) of migrating cells that expressed elevated c-src levels, elevated levels of the kinase negative mutant, and Neo<sup>r</sup> alone did not differ. The cells that expressed elevated levels of c-src appeared rounder and less flattened than the cells expressing Neo<sup>r</sup> alone, but their cytoplasmic areas were similar (552 $\pm$ 19 vs. 626 $\pm$ 63  $\mu$ m<sup>2</sup>, respectively, P = not significant).

The transformed endothelial cells were tested for expression of pp60<sup>c-src</sup>, production of c-src mRNA, and c-src kinase activity.

The cells transformed with the c-src gene expressed pp60<sup>c-src</sup> at higher levels than control BAEC cells (i.e., cells that had undergone no genetic engineering) as evidenced by the fact that the

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immunoblot assay showed evidence of pp60<sup>c-src</sup> after exposure of the XAR film for less than one day to extracts from transformed cells while extracts from control cells required a seven day exposure before evidence of this protein was seen.

The steady state src protein levels for the endothelial cells that expressed the kinase negative mutant were found to be even higher than those for the cells that expressed elevated levels of the c-src protein (i.e., on the order of 11 fold higher). However, no biologic effects were observed as a result of the expression of the kinase negative mutant src protein.

Consistent with the observed protein levels, the steady state level of the c-src retroviral mRNA transcript was fourfold greater in endothelial cells that expressed the kinase negative mutant than in cells that expressed elevated levels of wild-type c-src.

With regard to kinase activity, as shown in Figure 2, the in vitro src kinase activity was 2-3 fold greater in the cells that expressed elevated levels of c-src than in cells that expressed the kinase negative mutant or Neo<sup>r</sup> alone.

Significantly, with regard to the critical variable of cell migration, it was found that endothelial cells that expressed elevated levels of

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c-src migrated at a markedly faster rate in the fence assay than cells that expressed the kinase negative mutant, Neo<sup>r</sup> alone, or noninfected cells. The results of these experiments are shown in Figure 3, where each column (bar) represents the result of 8-10 replicates and the mean  $\pm$  1 SEM is shown. The difference between the c-src infected cells and the control cells is statistically significant at the 0.001 level.

As discussed above, the enhanced migration rates shown in Figure 3 mean that the genetically engineered endothelial cells of the present invention are superior to unmodified cells in terms of their ability to cover denuded sections of blood vessels and/or synthetic or natural grafts.

#### Example 2

##### Enhanced u-PA Activity of Genetically

##### Engineered Endothelial Cells

This example demonstrates the enhanced u-PA activity of endothelial cells which have been genetically engineered to express higher than normal levels of pp60<sup>c-src</sup>.

Control and genetically modified cells of the types described above in Example 1 were used for these experiments. u-PA activity was determined as described above.

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Figure 4 shows the results obtained. As shown therein, u-PA activity was significantly greater in endothelial cells that expressed elevated levels of c-src than in cells that expressed the kinase negative mutant, Neo<sup>r</sup> alone, or noninfected endothelial cells. Each column (bar) in Figure 4 represents the result of 5 replicates and the mean  $\pm$  1 SEM is shown. The difference between the c-src infected cells and the control cells was again statistically significant at the 0.001 level.

The enhanced production of u-PA is also illustrated by the photomicrographs of Figure 5. These photographs show the leading edge of the migrating endothelial cells after immunofluorescent staining as described above. Figure 5A shows cells expressing Neo<sup>r</sup> alone, while Figure 5B shows cells expressing elevated levels of c-src. As can be seen in this figure, the cells which have been genetically modified in accordance with the invention have significantly higher levels of u-PA production. As discussed above, this result means that these cells are superior to unmodified cells in terms of their ability to dissolve and/or prevent thrombus formation at the site of a surgical procedure.

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Example 3Interrelationship Between Enhanced  
u-PA Activity and Enhanced Migration

5 This example demonstrates that the enhanced  
migration achieved by the genetically engineered  
cells of the present invention is at least partially  
dependent on the enhanced u-PA production of those  
cells.

10 Antisera to bovine u-PA and nonimmune rabbit  
antisera were used to demonstrate the dependence.  
Migrating cells expressing elevated levels of  
pp60<sup>c-src</sup> and Neo<sup>r</sup> cells were exposed to the  
antibodies. The results are shown in Figure 6, where  
each column (bar) represents the result of 4  
15 replicates and the mean  $\pm$  1 SEM is shown.

The migration of the two cell types without  
antisera treatment are shown by the left most (solid)  
bars. As in Example 2, the c-src cells migrate  
significantly faster than the Neo<sup>r</sup> cells ( $P < 0.001$ ).  
20 Incubation with nonimmune rabbit antisera did not  
significantly change the migration of either cell  
type as shown by the middle (cross-hatched) bars.  
Incubation with anti-u-PA antisera, however, did  
significantly reduce the migration of the c-src cells  
25 ( $P < 0.01$ ) but not that of the Neo<sup>r</sup> cells as shown by  
the right most (open) bars.

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As this data shows, there is an interrelationship between the enhanced migration and enhanced u-PA activity exhibited by the genetically engineered cells of the invention in that binding of the u-PA by antibody diminishes the migration rate of the cells.

#### Example 4

##### Reduced Adherence of Monocytes to Genetically Engineered Endothelial Cells

This example demonstrates the reduced adherence of monocytes to endothelial cells which have been genetically engineered to express higher than normal levels of pp60<sup>c-src</sup>.

Using the adhesion assay described above, the adherence of U937 cells to migrating cultures of non-genetically engineered BAEC was determined. The monocytes were found to exhibit significant adherence to the monolayers of migrating BAEC, specifically to cells at or near the migratory front. The U937 cells adhered mainly around the periphery of the endothelial cells in this region, consistent with the loss of continuous cell-cell contact and changes in the expression of fibronectin in the zone of the migrating fronts. See Madri J.A., Pratt B.M., Yannariello-Brown J., "Matrix driven cell size changes modulate aortic endothelial cell proliferation and sheet migration," Amer. J. Pathol.

-51-

1988, 132:18-27; Madri J.A., Reidy M., Kocher O., Bell L., "Endothelial cell behavior following denudation injury is modulated by TGF- $\beta$  and fibronectin," Lab. Invest. 1989, 60:755-765; and  
5 DiCorleto P.E., De La Motte C.A., "Characterization of the adhesion of the human monocytic cell line U937 to cultured endothelial cells," J. Clin. Invest. 1985, 75: 1153-1161.

Stable overexpression of pp60<sup>c-src</sup> was found to  
10 decrease U937 cell adherence to migrating BAEC. Figure 7 illustrates this effect where the open bars represent the number of U937 cells bound to non-genetically engineered BAEC cells, the shaded bars, the number bound to BAEC cells expressing the  
15 Neo<sup>r</sup> gene, and the solid bars, the number bound to BAEC cells which overexpress pp60<sup>c-src</sup>.

As can be seen in this figure, overexpression of pp60<sup>c-src</sup> significantly decreases U937 cell adhesion to migrating BAEC ( $p < 0.05$ ). This decrease is  
20 manifest throughout the entire width of the migratory front that exhibits U937 adherence (-25% at 0.0 to 0.1 mm, -50% at 0.1 to 1.2 mm, -60% at 0.2 to 0.3 mm, and -60% at 0.3 mm to 0.4 mm).

The reduced monocyte adhesion shown by these  
25 experiments means that atherosclerotic plaques are less likely to occur in a vessel or graft containing the genetically engineered endothelial cells of the

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invention than in a vessel or graft containing non-genetically engineered endothelial cells since, as discussed above, the adherence of mononuclear cells to the endothelial lining of the vessel wall contributes to the atherosclerotic disease process.

#### Example 5

##### Reduced Fibronectin Production by

##### Genetically Engineered Endothelial Cells

This example demonstrates the reduced production of fibronectin by endothelial cells which have been genetically engineered to express higher than normal levels of pp60<sup>c-src</sup>.

Figure 8 shows the production of fibronectin, as determined by ELISA, of: 1) BAEC expressing only the neomycin resistance gene (neo<sup>r</sup>), and 2) BAEC expressing the neomycin resistance gene and overexpressing pp60<sup>c-src</sup> (c-src). As shown in this figure, migrating BAEC expressing the neomycin resistance gene and overexpressing pp60<sup>c-src</sup> exhibit significantly decreased steady state fibronectin levels ( $5.05 \pm 3.50 \mu\text{g/cell} \times 10^{-9}$ ) compared to migrating BAEC expressing only the neomycin resistance gene ( $35.50 \pm 1.90 \mu\text{g/cell} \times 10^{-9}$ ), a seven-fold decrease ( $p < 0.05$ ).

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Example 6Interrelationship Between  
Monocyte Adhesion and Fibronectin Production

5 This example demonstrates that the reduced  
adhesion of monocytes to the genetically engineered  
cells of the present invention is at least partially  
dependent upon the reduced production of fibronectin  
by those cells.

10 The experiments employed the cytokine TGF- $\beta$ 1  
which is known to induce fibronectin production by  
BAEC. See Madri J.A., Reidy M., Kocher O., Bell L.,  
"Endothelial cell behavior following denudation  
injury is modulated by TGF- $\beta$  and fibronectin," Lab.  
Invest. 1989, 60: 755-765. The TGF- $\beta$ 1 used in these  
15 tests was obtained from the National Cancer  
Institute, National Institutes of Health, Bethesda,  
Maryland.

In initial experiments, cultures of  
non-genetically engineered BAEC migrating on a  
20 laminin substrate were treated with TGF- $\beta$ 1 at a  
concentration of 0.5 ng/ml. This treatment resulted  
in a significant increase (70%) in adherence of U937  
cells to the migrating fronts of the monolayers of  
BAEC.

25 TGF- $\beta$ 1 treatment of genetically engineered BAEC  
was similarly found to result in an increase in U937  
cell adherence at or near the migrating front.

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Specifically, when treated with 0.5 ng/ml TGF- $\beta$ 1, BAEC which express the neomycin resistance gene and overexpress pp60<sup>c-src</sup> exhibit increased adherence for U937 cells similar to that observed in non-genetically engineered BAEC treated with the same concentration of TGF- $\beta$ 1.

The results of these experiments are shown in Figure 9 wherein the open bars represent the reduced adherence achieved by the BAEC which overexpress pp60<sup>c-src</sup> and the open bars represent the increase in adherence resulting from the treatment of these cells with TGF- $\beta$ 1. As illustrated in this figure, a 150% increase in U937 cell adhesion results from the TGF- $\beta$ 1 treatment of the pp60<sup>c-src</sup> and neomycin resistance gene expressing cells ( $p < 0.05$ ). Immunofluorescence using antibodies directed against fibronectin revealed that pp60<sup>c-src</sup> transfected BAEC treated with TGF- $\beta$ 1 synthesized and deposited increased amounts of fibronectin into the cell layer compared to untreated cells.

As this data shows, there is an interrelationship between the reduced adhesion of monocytes to the genetically engineered endothelial cells of the invention and the reduced production of fibronectin by those cells.

A variety of modifications which do not depart from the scope and spirit of the invention will be

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evident to persons of ordinary skill in the art from  
the disclosure herein. The following claims are  
intended to cover the specific embodiments described  
herein as well as such modifications, variations, and  
5 equivalents.

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20

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SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Bell, Leonard  
Madri, Joseph A.  
Warren, Stephen L.  
Luthringer, Daniel J.
- (ii) TITLE OF INVENTION: Genetically Engineered  
Endothelial Cells
- (iii) NUMBER OF SEQUENCES: 4
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  - (F) ZIP: 06430
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: 3.5 inch, 760 Kb storage
  - (B) COMPUTER: DELL 486/50
  - (C) OPERATING SYSTEM: DOS 5.0
  - (D) SOFTWARE: Displaywrite 3
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE: 05-JAN-1993
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: 07/820,011
  - (B) FILING DATE: 06-JAN-1992

-57-

(viii) ATTORNEY/AGENT INFORMATION:

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- (B) REGISTRATION NUMBER: 30,399
- (C) REFERENCE/DOCKET NUMBER: ALX-101PCT

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-58-

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1602 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Gallus, gallus

## (x) PUBLICATION INFORMATION:

- (A) AUTHORS: Takeya, Tatsuo  
Hanafusa, Hidesaburo
- (B) TITLE: Structure and Sequence of the  
Cellular Gene Homologous to the RSV src  
Gene and the Mechanism for Generating the  
Transforming Virus
- (C) JOURNAL: Cell
- (D) VOLUME: 32
- (F) PAGES: 881-890
- (G) DATE: March, 1983

-59-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATG GGG AGC AGC AAG AGC AAG CCC AAG GAC CCC AGC CAG CGC CGG CGC	48
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20 25 30	
CAG ACC CCC AAC AAG ACA GCA GCC CCC GAC ACG CAC CGC ACC CCC AGC	144
Gln Thr Pro Asn Lys Thr Ala Ala Pro Asp Thr His Arg Thr Pro Ser	
35 40 45	
CGC TCC TTT GGG ACC GTG GCC ACC GAG CCC AAG CTC TTC GGG GGC TTC	192
Arg Ser Phe Gly Thr Val Ala Thr Glu Pro Lys Leu Phe Gly Gly Phe	
50 55 60	
AAC ACT TCT GAC ACC GTT ACG TCG CCG CAG CGT GCC GGG GCA CTG GCT	240
Asn Thr Ser Asp Thr Val Thr Ser Pro Gln Arg Ala Gly Ala Leu Ala	
65 70 75 80	
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Gly Gly Val Thr Thr Phe Val Ala Leu Tyr Asp Tyr Glu Ser Arg Thr	
85 90 95	
GAA ACG GAC TTG TCC TTC AAG AAA GGA GAA CGC CTG CAG ATT GTC AAC	336
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100 105 110	
AAC ACG GAA GGT GAC TGG TGG CTG GCT CAT TCC CTC ACT ACA GGA CAG	384
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115 120 125	
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130 135 140	
GCT GAA GAG TGG TAC TTT GGG AAG ATC ACT CGT CGG GAG TCC GAG CGG	480
Ala Glu Glu Trp Tyr Phe Gly Lys Ile Thr Arg Arg Glu Ser Glu Arg	
145 150 155 160	

-60-

CTG	CTG	CTC	AAC	CCC	GAA	AAC	CCC	CGG	GGA	ACC	TTC	TTG	GTC	CGG	GAG	528
Leu	Leu	Leu	Asn	Pro	Glu	Asn	Pro	Arg	Gly	Thr	Phe	Leu	Val	Arg	Glu	
			165						170					175		
AGC	GAG	ACG	ACA	AAA	GGT	GCC	TAT	TGC	CTC	TCC	GTT	TCT	GAC	TTT	GAC	576
Ser	Glu	Thr	Thr	Lys	Gly	Ala	Tyr	Cys	Leu	Ser	Val	Ser	Asp	Phe	Asp	
			180					185					190			
AAC	GCC	AAG	GGG	CTC	AAT	GTG	AAG	CAC	TAC	AAG	ATC	CGC	AAG	CTG	GAC	624
Asn	Ala	Lys	Gly	Leu	Asn	Val	Lys	His	Tyr	Lys	Ile	Arg	Lys	Leu	Asp	
		195					200					205				
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Ser	Gly	Gly	Phe	Tyr	Ile	Thr	Ser	Arg	Thr	Gln	Phe	Ser	Ser	Leu	Gln	
	210					215					220					
CAG	CTG	GTG	GCC	TAC	TAC	TCC	AAA	CAT	GCT	GAT	GGC	TTG	TGC	CAC	CGC	720
Gln	Leu	Val	Ala	Tyr	Tyr	Ser	Lys	His	Ala	Asp	Gly	Leu	Cys	His	Arg	
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CTG	ACC	AAC	GTC	TGC	CCC	ACG	TCC	AAG	CCC	CAG	ACC	CAG	GGA	CTC	GCC	768
Leu	Thr	Asn	Val	Cys	Pro	Thr	Ser	Lys	Pro	Gln	Thr	Gln	Gly	Leu	Ala	
			245						250					255		
AAG	GAC	GCG	TGG	GAA	ATC	CCC	CGG	GAG	TCG	CTG	CGG	CTG	GAG	GTG	AAG	816
Lys	Asp	Ala	Trp	Glu	Ile	Pro	Arg	Glu	Ser	Leu	Arg	Leu	Glu	Val	Lys	
		260						265					270			
CTG	GGG	CAG	GGC	TGC	TTT	GGA	GAG	GTC	TGG	ATG	GGG	ACC	TGG	AAC	GGC	864
Leu	Gly	Gln	Gly	Cys	Phe	Gly	Glu	Val	Trp	Met	Gly	Thr	Trp	Asn	Gly	
	275					280						285				
ACC	ACC	AGA	GTG	GCC	ATA	AAG	ACT	CTG	AAG	CCC	GGC	AAC	ATG	TCC	CCG	912
Thr	Thr	Arg	Val	Ala	Ile	Lys	Thr	Leu	Lys	Pro	Gly	Thr	Met	Ser	Pro	
	290					295					300					
GAG	GCC	TTC	CTG	CAG	GAA	GCC	CAA	GTG	ATG	AAG	AAG	CTC	CGG	CAT	GAG	960
Glu	Ala	Phe	Leu	Gln	Glu	Ala	Gln	Val	Met	Lys	Lys	Leu	Arg	His	Glu	
305				310						315					320	
AAG	CTG	GTT	CAG	CTG	TAC	GCA	GTG	GTG	TCG	GAA	GAG	CCC	ATC	TAC	ATC	1008
Lys	Leu	Val	Gln	Leu	Tyr	Ala	Val	Val	Ser	Glu	Glu	Pro	Ile	Tyr	Ile	
			325						330					335		

-61-

GTC ACT GAG TAC ATG AGC AAG GGG AGC CTC CTG GAT TTC CTG AAG GGA Val Thr Glu Tyr Met Ser Lys Gly Ser Leu Leu Asp Phe Leu Lys Gly 340 345 350	1056
GAG ATG GGC AAG TAC CTG CGG CTG CCA CAG CTC GTC GAT ATG GCT GCT Glu Met Gly Lys Tyr Leu Arg Leu Pro Gln Leu Val Asp Met Ala Ala 355 360 365	1104
CAG ATT GCA TCC GGC ATG GCC TAT GTG GAG AGG ATG AAC TAC GTG CAC Gln Ile Ala Ser Gly Met Ala Tyr Val Glu Arg Met Asn Tyr Val His 370 375 380	1152
CGA GAC CTG CGG GCG GCC AAC ATC CTG GTG GGG GAG AAC CTG GTG TGC Arg Asp Leu Arg Ala Ala Asn Ile Leu Val Gly Glu Asn Leu Val Cys 385 390 395 400	1200
AAG GTG GCT GAC TTT GGG CTG GCA CGC CTC ATC GAG GAC AAC GAG TAC Lys Val Ala Asp Phe Gly Leu Ala Arg Leu Ile Glu Asp Asn Glu Tyr 405 410 415	1248
ACA GCA CGG CAA GGT GCC AAG TTC CCC ATC AAG TGG ACA GCC CCC GAG Thr Ala Arg Gln Gly Ala Lys Phe Pro Ile Lys Trp Thr Ala Pro Glu 420 425 430	1296
GCA GCC CTC TAT GGC CGG TTC ACC ATC AAG TCG GAT GTC TGG TCC TTC Ala Ala Leu Tyr Gly Arg Phe Thr Ile Lys Ser Asp Val Trp Ser Phe 435 440 445	1344
GGC ATC CTG CTG ACT GAG CTG ACC ACC AAG GGC CGG GTG CCA TAC CCA Gly Ile Leu Leu Thr Glu Leu Thr Thr Lys Gly Arg Val Pro Tyr Pro 450 455 460	1392
GGG ATG GTC AAC AGG GAG GTG CTG GAC CAG GTG GAG AGG GGC TAC CGC Gly Met Val Asn Arg Glu Val Leu Asp Gln Val Glu Arg Gly Tyr Arg 465 470 475 480	1440
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CAG	GCC	TTC	CTG	GAG	GAC	TAC	TTC	ACC	TCG	ACA	GAG	CCC	CAG	TAC	CAG	1584
Gln	Ala	Phe	Leu	Glu	Asp	Tyr	Phe	Thr	Ser	Thr	Glu	Pro	Glu	Tyr	Gln	
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CCT	GGA	GAG	AAC	CTA	TAG	1602
Pro	Gly	Glu	Asn	Leu		
530						

-63-

## (3) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 533 amino acids

(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Protein

(iii) HYPOTHETICAL: No

(v) FRAGMENT TYPE: Complete Sequence

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Gallus, gallus

## (x) PUBLICATION INFORMATION:

(A) AUTHORS: Takeya, Tatsuo  
Hanafusa, Hidesaburo(B) TITLE: Structure and Sequence of the  
Cellular Gene Homologous to the RSV src  
Gene and the Mechanism for Generating the  
Transforming Virus

(C) JOURNAL: Cell

(D) VOLUME: 32

(F) PAGES: 881-890

(G) DATE: March, 1983

-64-

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Gly Ser Ser Lys Ser Lys Pro Lys Asp Pro Ser Gln Arg Arg Arg  
5 10 15

Ser Leu Glu Pro Pro Asp Ser Thr His His Gly Gly Phe Pro Ala Ser  
20 25 30

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35 40 45

Arg Ser Phe Gly Thr Val Ala Thr Glu Pro Lys Leu Phe Gly Gly Phe  
50 55 60

Asn Thr Ser Asp Thr Val Thr Ser Pro Gln Arg Ala Gly Ala Leu Ala  
65 70 75 80

Gly Gly Val Thr Thr Phe Val Ala Leu Tyr Asp Tyr Glu Ser Arg Thr  
85 90 95

Glu Thr Asp Leu Ser Phe Lys Lys Gly Glu Arg Leu Gln Ile Val Asn  
100 105 110

Asn Thr Glu Gly Asp Trp Trp Leu Ala His Ser Leu Thr Thr Gly Gln  
115 120 125

Thr Gly Tyr Ile Pro Ser Asn Tyr Val Ala Pro Ser Asp Ser Ile Gln  
130 135 140

Ala Glu Glu Trp Tyr Phe Gly Lys Ile Thr Arg Arg Glu Ser Glu Arg  
145 150 155 160

Leu Leu Leu Asn Pro Glu Asn Pro Arg Gly Thr Phe Leu Val Arg Glu  
165 170 175

Ser Glu Thr Thr Lys Gly Ala Tyr Cys Leu Ser Val Ser Asp Phe Asp  
180 185 190

-65-

Asn Ala Lys Gly Leu Asn Val Lys His Tyr Lys Ile Arg Lys Leu Asp  
 195 200 205

Ser Gly Gly Phe Tyr Ile Thr Ser Arg Thr Gln Phe Ser Ser Leu Gln  
 210 215 220

Gln Leu Val Ala Tyr Tyr Ser Lys His Ala Asp Gly Leu Cys His Arg  
 225 230 235 240

Leu Thr Asn Val Cys Pro Thr Ser Lys Pro Gln Thr Gln Gly Leu Ala  
 245 250 255

Lys Asp Ala Trp Glu Ile Pro Arg Glu Ser Leu Arg Leu Glu Val Lys  
 260 265 270

Leu Gly Gln Gly Cys Phe Gly Glu Val Trp Met Gly Thr Trp Asn Gly  
 275 280 285

Thr Thr Arg Val Ala Ile Lys Thr Leu Lys Pro Gly Thr Met Ser Pro  
 290 295 300

Glu Ala Phe Leu Gln Glu Ala Gln Val Met Lys Lys Leu Arg His Glu  
 305 310 315 320

Lys Leu Val Gln Leu Tyr Ala Val Val Ser Glu Glu Pro Ile Tyr Ile  
 325 330 335

Val Thr Glu Tyr Met Ser Lys Gly Ser Leu Leu Asp Phe Leu Lys Gly  
 340 345 350

Glu Met Gly Lys Tyr Leu Arg Leu Pro Gln Leu Val Asp Met Ala Ala  
 355 360 365

Gln Ile Ala Ser Gly Met Ala Tyr Val Glu Arg Met Asn Tyr Val His  
 370 375 380

Arg Asp Leu Arg Ala Ala Asn Ile Leu Val Gly Glu Asn Leu Val Cys  
 385 390 395 400

Lys Val Ala Asp Phe Gly Leu Ala Arg Leu Ile Glu Asp Asn Glu Tyr  
 405 410 415

-66-

Thr Ala Arg Gln Gly Ala Lys Phe Pro Ile Lys Trp Thr Ala Pro Glu  
420 425 430

Ala Ala Leu Tyr Gly Arg Phe Thr Ile Lys Ser Asp Val Trp Ser Phe  
435 440 445

Gly Ile Leu Leu Thr Glu Leu Thr Thr Lys Gly Arg Val Pro Tyr Pro  
450 455 460

Gly Met Val Asn Arg Glu Val Leu Asp Gln Val Glu Arg Gly Tyr Arg  
465 470 475 480

Met Pro Cys Pro Pro Glu Cys Pro Glu Ser Leu His Asp Leu Met Cys  
485 490 495

Gln Cys Trp Arg Arg Asp Pro Glu Glu Arg Pro Thr Phe Glu Tyr Leu  
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Gln Ala Phe Leu Glu Asp Tyr Phe Thr Ser Thr Glu Pro Glu Tyr Gln  
515 520 525

Pro Gly Glu Asn Leu  
530

-67-

## (4) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1611
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapien

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: Chromosome 20

## (x) PUBLICATION INFORMATION:

(A) AUTHORS: Anderson, Stephen K.  
Gibbs, Carol P.  
Tanaka, Akio  
Kung, Hsing-Jien  
Fujita, Donald J.

(B) TITLE: Human Cellular src Gene:  
Nucleotide Sequence and Derived Amino  
Acid Sequence of the Region Coding for  
the Carboxy-Terminal Two-Thirds of  
pp60<sup>c</sup>-src

(C) JOURNAL: Molecular and Cellular Biology

(D) VOLUME: 5

(E) ISSUE: 5

(F) PAGES: 1122-1129

(G) DATE: May, 1985

## (x) PUBLICATION INFORMATION:

(A) AUTHORS: Tanaka, Akio  
Gibbs, Carol P.

-68-

Arthur, Richard R.  
Anderson, Stephen K.  
Kung, Hsing-Jien  
Fujita, Donald J.

- (B) TITLE: DNA Sequence Encoding the  
Amino-Terminal Region of the Human c-src  
Protein: Implications of Sequence  
Divergence among src-Type Kinase  
Oncogenes
- (C) JOURNAL: Molecular and Cellular Biology
- (D) VOLUME: 7
- (E) ISSUE: 5
- (F) PAGES: 1978-1983
- (G) DATE: May, 1987

- 69 -

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATG GGT AGC AAC AAG AGC AAG CCC AAG GAT GCC AGC CAG CGG CGC CGC	48
Met Gly Ser Asn Lys Ser Lys Pro Lys Asp Ala Ser Gln Arg Arg Arg	
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AGC CTG GAG CCC GCC GAG AAC GTG CAC GGC GCT GGC GGG GGC GCT TTC	96
Ser Leu Glu Pro Ala Glu Asn Val His Gly Ala Gly Gly Gly Ala Phe	
20 25 30	
CCC GCC TCG CAG ACC CCC AGC AAG CCA GCC TCG GCC GAC GGC CAC CGC	144
Pro Ala Ser Gln Thr Pro Ser Lys Pro Ala Ser Ala Asp Gly His Arg	
35 40 45	
GGC CCC AGC GCG GCC TTC GCC CCC GCG GCC GCC GAG CCC AAG CTG TTC	192
Gly Pro Ser Ala Ala Phe Ala Pro Ala Ala Ala Glu Pro Lys Leu Phe	
50 55 60	
GGA GGC TTC AAC TCC TCG GAC ACC GTC ACC TCC CCG CAG AGG GCG GGC	240
Gly Gly Phe Asn Ser Ser Asp Thr Val Thr Ser Pro Gln Arg Ala Gly	
65 70 75 80	
CCG CTG GCC GGT GGA GTG ACC ACC TTT GTG GCC CTC TAT GAC TAT GAG	288
Pro Leu Ala Gly Gly Val Thr Thr Phe Val Ala Leu Tyr Asp Tyr Glu	
85 90 95	
TCT AGG ACG GAG ACA GAC CTG TCC TTC AAG AAA GGC GAG CGG CTC CAG	336
Ser Arg Thr Glu Thr Asp Leu Ser Phe Lys Lys Gly Glu Arg Leu Gln	
100 105 110	
ATT GTC AAC AAC ACA GAG GGA GAC TGG TGG CTG GCC CAC TCG CTC AGC	384
Ile Val Asn Asn Thr Glu Gly Asp Trp Trp Leu Ala His Ser Leu Ser	
115 120 125	
ACA GGA CAG ACA GGC TAC ATC CCC AGC AAC TAC GTG GCG CCC TCC GAC	432
Thr Gly Gln Thr Gly Tyr Ile Pro Ser Asn Tyr Val Ala Pro Ser Asp	
130 135 140	
TCC ATC CAG GCT GAG GAG TGG TAT TTT GGC AAG ATC ACC AGA CGG GAG	480
Ser Ile Gln Ala Glu Glu Trp Tyr Phe Gly Lys Ile Thr Arg Arg Glu	
145 150 155 160	

-70-

TCA	GAG	CGG	TTA	CTG	CTC	AAT	GCA	GAG	AAC	CCG	AGA	GGG	ACC	TTC	CTC	528
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			165						170					175		
GTG	CGA	GAA	AGT	GAG	ACC	ACG	AAA	GGT	GCC	TAC	TGC	CTC	TCA	GTG	TCT	576
Val	Arg	Glu	Ser	Glu	Thr	Thr	Lys	Gly	Ala	Tyr	Cys	Leu	Ser	Val	Ser	
			180					185					190			
GAC	TTC	GAC	AAC	GCC	AAG	GGC	CTC	AAC	GTG	AAG	CAC	TAC	AAG	ATC	CGC	624
Asp	Phe	Asp	Asn	Ala	Lys	Gly	Leu	Asn	Val	Lys	His	Tyr	Lys	Ile	Arg	
		195					200					205				
AAG	CTG	GAC	AGC	GGC	GGC	TTC	TAC	ATC	ACC	TCC	CGC	ACC	CAG	TTC	AAC	672
Lys	Leu	Asp	Ser	Gly	Gly	Phe	Tyr	Ile	Thr	Ser	Arg	Thr	Gln	Phe	Asn	
	210					215					220					
AGC	CTG	CAG	CAG	CTG	GTG	GCC	TAC	TAC	TCC	AAA	CAC	GCC	GAT	GGC	CTG	720
Ser	Leu	Gln	Gln	Leu	Val	Ala	Tyr	Tyr	Ser	Lys	His	Ala	Asp	Gly	Leu	
225					230					235					240	
TGC	CAC	CGC	CTC	ACC	ACC	GTG	TGC	CCC	ACG	TCC	AAG	CCG	CAG	ACT	CAG	768
Cys	His	Arg	Leu	Thr	Thr	Val	Cys	Pro	Thr	Ser	Lys	Pro	Gln	Thr	Gln	
				245					250					255		
GGC	CTG	GCC	AAG	GAT	GCC	TGG	GAG	ATC	CCT	CGG	GAG	TCG	CTG	CGG	CTG	816
Gly	Leu	Ala	Lys	Asp	Ala	Trp	Glu	Ile	Pro	Arg	Glu	Ser	Leu	Arg	Leu	
			260					265					270			
GAG	GTC	AAG	CTG	GGC	CAG	GGC	TGC	TTT	GGC	GAG	GTG	TGG	ATG	GGG	ACC	864
Glu	Val	Lys	Leu	Gly	Gln	Gly	Cys	Phe	Gly	Glu	Val	Trp	Met	Gly	Thr	
		275					280					285				
TGG	AAC	GGT	ACC	ACC	AGG	GTG	GCC	ATC	AAA	ACC	CTG	AAG	CCT	GGC	ACG	912
Trp	Asn	Gly	Thr	Thr	Arg	Val	Ala	Ile	Lys	Thr	Leu	Lys	Pro	Gly	Thr	
	290					295					300					
ATG	TCT	CCA	GAG	GCC	TTC	CTG	CAG	GAG	GCC	CAG	GTC	ATG	AAG	AAG	CTG	960
Met	Ser	Pro	Glu	Ala	Phe	Leu	Gln	Glu	Ala	Gln	Val	Met	Lys	Lys	Leu	
305					310				315						320	
AGG	CAT	GAG	AAG	CTG	GTG	CAG	TTG	TAT	GCT	GTG	GTT	TCA	GAG	GAG	CCC	1008
Arg	His	Glu	Lys	Leu	Val	Gln	Leu	Tyr	Ala	Val	Val	Ser	Glu	Glu	Pro	
				325					330					335		

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ATT TAC ATC GTC ACG GAG TAC ATG AGC AAG GGG AGT TTG CTG GAC TTT Ile Tyr Ile Val Thr Glu Tyr Met Ser Lys Gly Ser Leu Leu Asp Phe 340 345 350	1056
CTC AAG GGG GAG ACA GGC AAG TAC CTG CGG CTG CCT CAG CTG GTG GAC Leu Lys Gly Glu Thr Gly Lys Tyr Leu Arg Leu Pro Gln Leu Val Asp 355 360 365	1104
ATG GCT GCT CAG ATC GCC TCA GGC ATG GCG TAC GTG GAG CGG ATG AAC Met Ala Ala Gln Ile Ala Ser Gly Met Ala Tyr Val Glu Arg Met Asn 370 375 380	1152
TAC GTC CAC CGG GAC CTT CGT GCA GCC AAC ATC CTG GTG GGA GAG AAC Tyr Val His Arg Asp Leu Arg Ala Ala Asn Ile Leu Val Gly Glu Asn 385 390 395 400	1200
CTG GTG TGC AAA GTG GCC GAC TTT GGG CTG GCT CGG CTC ATT GAA GAC Leu Val Cys Lys Val Ala Asp Phe Gly Leu Ala Arg Leu Ile Glu Asp 405 410 415	1248
AAT GAG TAC ACG GCG CGG CAA GGT GCC AAA TTC CCC ATC AAG TGG ACG Asn Glu Tyr Thr Ala Arg Gln Gly Ala Lys Phe Pro Ile Lys Trp Thr 420 425 430	1296
GCT CCA GAA GCT GCC CTC TAT GGC CGC TTC ACC ATC AAG TCG GAC GTG Ala Pro Glu Ala Ala Leu Tyr Gly Arg Phe Thr Ile Lys Ser Asp Val 435 440 445	1344
TGG TCC TTC GGG ATC CTG CTG ACT GAG CTC ACC ACA AAG GGA CGG GTG Trp Ser Phe Gly Ile Leu Leu Thr Glu Leu Thr Thr Lys Gly Arg Val 450 455 460	1392
CCC TAC CCT GGG ATG GTG AAC CGC GAG GTG CTG GAC CAG GTG GAG CGG Pro Tyr Pro Gly Met Val Asn Arg Glu Val Leu Asp Gln Val Glu Arg 465 470 475 480	1440
GGC TAC CGG ATG CCC TGC CCG CCG GAG TGT CCC GAG TCC CTG CAC GAC Gly Tyr Arg Met Pro Cys Pro Pro Glu Cys Pro Glu Ser Leu His Asp 485 490 495	1488
CTC ATG TGC CAG TGC TGG CGG AAG GAG CCT GAG GAG CGG CCC ACC TTC Leu Met Cys Gln Cys Trp Arg Lys Glu Pro Glu Glu Arg Pro Thr Phe 500 505 510	1536

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GAG	TAC	CTG	CAG	GCC	TTC	CTG	GAG	GAC	TAC	TTC	ACG	TCC	ACC	GAG	CCC	1584
Glu	Tyr	Leu	Gln	Ala	Phe	Leu	Glu	Asp	Tyr	Phe	Thr	Ser	Thr	Glu	Pro	
		515					520					525				

CAG TAC CAG CCC GGG GAG AAC CTC TAG 1611  
Gln Tyr Gln Pro Gly Glu Asn Leu  
530 535

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## (5) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 536 amino acids

(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Protein

(iii) HYPOTHETICAL: No

(v) FRAGMENT TYPE: Complete Sequence

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapien

## (x) PUBLICATION INFORMATION:

(A) AUTHORS: Anderson, Stephen K.  
Gibbs, Carol P.  
Tanaka, Akio  
Kung, Hsing-Jien  
Fujita, Donald J.

(B) TITLE: Human Cellular src Gene:  
Nucleotide Sequence and Derived Amino  
Acid Sequence of the Region Coding for  
the Carboxy-Terminal Two-Thirds of  
pp60<sup>c</sup>-src

(C) JOURNAL: Molecular and Cellular Biology

(D) VOLUME: 5

(E) ISSUE: 5

(F) PAGES: 1122-1129

(G) DATE: May, 1985

## (x) PUBLICATION INFORMATION:

(A) AUTHORS: Tanaka, Akio  
Gibbs, Carol P.  
Arthur, Richard R.  
Anderson, Stephen K.  
Kung, Hsing-Jien  
Fujita, Donald J.

-74-

- (B) TITLE: DNA Sequence Encoding the  
Amino-Terminal Region of the Human c-src  
Protein: Implications of Sequence  
Divergence among src-Type Kinase  
Oncogenes
- (C) JOURNAL: Molecular and Cellular Biology
- (D) VOLUME: 7
- (E) ISSUE: 5
- (F) PAGES: 1978-1983
- (G) DATE: May, 1987

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Gly Ser Asn Lys Ser Lys Pro Lys Asp Ala Ser Gln Arg Arg Arg  
                                   5                                  10                                  15

Ser Leu Glu Pro Ala Glu Asn Val His Gly Ala Gly Gly Gly Ala Phe  
                                   20                                  25                                  30

Pro Ala Ser Gln Thr Pro Ser Lys Pro Ala Ser Ala Asp Gly His Arg  
                                   35                                  40                                  45

Gly Pro Ser Ala Ala Phe Ala Pro Ala Ala Ala Glu Pro Lys Leu Phe  
                                   50                                  55                                  60

Gly Gly Phe Asn Ser Ser Asp Thr Val Thr Ser Pro Gln Arg Ala Gly  
                                   65                                  70                                  75                                  80

Pro Leu Ala Gly Gly Val Thr Thr Phe Val Ala Leu Tyr Asp Tyr Glu  
                                   85                                  90                                  95

Ser Arg Thr Glu Thr Asp Leu Ser Phe Lys Lys Gly Glu Arg Leu Gln  
                                   100                                  105                                  110

Ile Val Asn Asn Thr Glu Gly Asp Trp Trp Leu Ala His Ser Leu Ser  
                                   115                                  120                                  125

Thr Gly Gln Thr Gly Tyr Ile Pro Ser Asn Tyr Val Ala Pro Ser Asp  
                                   130                                  135                                  140

Ser Ile Gln Ala Glu Glu Trp Tyr Phe Gly Lys Ile Thr Arg Arg Glu  
                                   145                                  150                                  155                                  160

Ser Glu Arg Leu Leu Leu Asn Ala Glu Asn Pro Arg Gly Thr Phe Leu  
                                   165                                  170                                  175

Val Arg Glu Ser Glu Thr Thr Lys Gly Ala Tyr Cys Leu Ser Val Ser  
                                   180                                  185                                  190

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Asp Phe Asp Asn Ala Lys Gly Leu Asn Val Lys His Tyr Lys Ile Arg  
 195 200 205

Lys Leu Asp Ser Gly Gly Phe Tyr Ile Thr Ser Arg Thr Gln Phe Asn  
 210 215 220

Ser Leu Gln Gln Leu Val Ala Tyr Tyr Ser Lys His Ala Asp Gly Leu  
 225 230 235 240

Cys His Arg Leu Thr Thr Val Cys Pro Thr Ser Lys Pro Gln Thr Gln  
 245 250 255

Gly Leu Ala Lys Asp Ala Trp Glu Ile Pro Arg Glu Ser Leu Arg Leu  
 260 265 270

Glu Val Lys Leu Gly Gln Gly Cys Phe Gly Glu Val Trp Met Gly Thr  
 275 280 285

Trp Asn Gly Thr Thr Arg Val Ala Ile Lys Thr Leu Lys Pro Gly Thr  
 290 295 300

Met Ser Pro Glu Ala Phe Leu Gln Glu Ala Gln Val Met Lys Lys Leu  
 305 310 315 320

Arg His Glu Lys Leu Val Gln Leu Tyr Ala Val Val Ser Glu Glu Pro  
 325 330 335

Ile Tyr Ile Val Thr Glu Tyr Met Ser Lys Gly Ser Leu Leu Asp Phe  
 340 345 350

Leu Lys Gly Glu Thr Gly Lys Tyr Leu Arg Leu Pro Gln Leu Val Asp  
 355 360 365

Met Ala Ala Gln Ile Ala Ser Gly Met Ala Tyr Val Glu Arg Met Asn  
 370 375 380

Tyr Val His Arg Asp Leu Arg Ala Ala Asn Ile Leu Val Gly Glu Asn  
 385 390 395 400

Leu Val Cys Lys Val Ala Asp Phe Gly Leu Ala Arg Leu Ile Glu Asp  
 405 410 415

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Asn Glu Tyr Thr Ala Arg Gln Gly Ala Lys Phe Pro Ile Lys Trp Thr  
420 425 430

Ala Pro Glu Ala Ala Leu Tyr Gly Arg Phe Thr Ile Lys Ser Asp Val  
435 440 445

Trp Ser Phe Gly Ile Leu Leu Thr Glu Leu Thr Thr Lys Gly Arg Val  
450 455 460

Pro Tyr Pro Gly Met Val Asn Arg Glu Val Leu Asp Gln Val Glu Arg  
465 470 475 480

Gly Tyr Arg Met Pro Cys Pro Pro Glu Cys Pro Glu Ser Leu His Asp  
485 490 495

Leu Met Cys Gln Cys Trp Arg Lys Glu Pro Glu Glu Arg Pro Thr Phe  
500 505 510

Glu Tyr Leu Gln Ala Phe Leu Glu Asp Tyr Phe Thr Ser Thr Glu Pro  
515 520 525

Gln Tyr Gln Pro Gly Glu Asn Leu  
530 535

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What is claimed is:

1. Endothelial cells transformed with a heterologous vector containing DNA coding for the c-src polypeptide.

2. The endothelial cells of Claim 1 wherein the cells exhibit enhanced migration in comparison to cells which have not been so transformed.

3. The endothelial cells of Claim 1 wherein the cells exhibit enhanced urokinase plasminogen activator activity in comparison to cells which have not been so transformed.

4. The endothelial cells of Claim 1 wherein the cells produce enhanced amounts of tyrosine kinase in comparison to cells which have not been so transformed.

5. The endothelial cells of Claim 1 wherein the cells exhibit enhanced tyrosine kinase activity in comparison to cells which have not been so transformed.

6. The endothelial cells of Claim 1 wherein the cells exhibit reduced monocyte adhesion in comparison to cells which have not been so transformed.

7. The endothelial cells of Claim 1 wherein the cells exhibit reduced fibronectin production in comparison to cells which have not been so transformed.

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8. The endothelial cells of Claim 1 wherein the DNA coding for the c-src polypeptide is operably linked to a heterologous promoter.

9. The endothelial cells of Claim 1 wherein the cells are human endothelial cells.

10. Endothelial cells transformed with a heterologous vector containing DNA coding for at least a portion of the c-src polypeptide, said cells exhibiting enhanced migration in comparison to cells which have not been so transformed.

11. Endothelial cells transformed with a heterologous vector containing DNA coding for at least a portion of the c-src polypeptide, said cells exhibiting enhanced urokinase plasminogen activator activity in comparison to cells which have not been so transformed.

12. Endothelial cells transformed with a heterologous vector containing DNA coding for at least a portion of the c-src polypeptide, said cells exhibiting reduced monocyte adhesion in comparison to cells which have not been so transformed.

13. Endothelial cells transformed with a heterologous vector containing DNA coding for at least a portion of the c-src polypeptide, said cells exhibiting reduced fibronectin production in comparison to cells which have not been so transformed.

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14. A prosthesis for implantation in the vascular system of an animal comprising a solid support carrying endothelial cells transformed with a heterologous vector containing DNA coding for the c-src polypeptide.

15. The prosthesis of Claim 14 wherein the solid support comprises an autologous vascular graft.

16. The prosthesis of Claim 14 wherein the solid support comprises a synthetic vascular graft.

17. The prosthesis of Claim 14 wherein the solid support comprises a stent.

18. The prosthesis of Claim 14 wherein the endothelial cells exhibit enhanced migration in comparison to cells which have not been so transformed.

19. The prosthesis of Claim 14 wherein the endothelial cells exhibit enhanced urokinase plasminogen activator activity in comparison to cells which have not been so transformed.

20. The prosthesis of Claim 14 wherein the endothelial cells produce enhanced amounts of tyrosine kinase in comparison to cells which have not been so transformed.

21. The prosthesis of Claim 14 wherein the endothelial cells exhibit enhanced tyrosine kinase activity in comparison to cells which have not been so transformed.

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22. The prosthesis of Claim 14 wherein the endothelial cells exhibit reduced monocyte adhesion in comparison to cells which have not been so transformed.

23. The prosthesis of Claim 14 wherein the endothelial cells exhibit reduced fibronectin production in comparison to cells which have not been so transformed.

24. The prosthesis of Claim 14 wherein the the DNA coding for the c-src polypeptide is operably linked to a heterologous promoter.

25. The prosthesis of Claim 14 wherein the endothelial cells are human endothelial cells.

26. A prosthesis for implantation in the vascular system of an animal comprising a solid support carrying endothelial cells transformed with a heterologous vector containing DNA coding for at least a portion of the c-src polypeptide, said endothelial cells exhibiting enhanced migration in comparison to cells which have not been so transformed.

27. A prosthesis for implantation in the vascular system of an animal comprising a solid support carrying endothelial cells transformed with a heterologous vector containing DNA coding for at least a portion of the c-src polypeptide, said endothelial cells exhibiting enhanced urokinase

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plasminogen activator activity in comparison to cells which have not been so transformed.

28. A prosthesis for implantation in the vascular system of an animal comprising a solid support carrying endothelial cells transformed with a heterologous vector containing DNA coding for at least a portion of the c-src polypeptide, said endothelial cells exhibiting reduced monocyte adhesion in comparison to cells which have not been so transformed.

29. A prosthesis for implantation in the vascular system of an animal comprising a solid support carrying endothelial cells transformed with a heterologous vector containing DNA coding for at least a portion of the c-src polypeptide, said endothelial cells exhibiting reduced fibronectin production in comparison to cells which have not been so transformed.

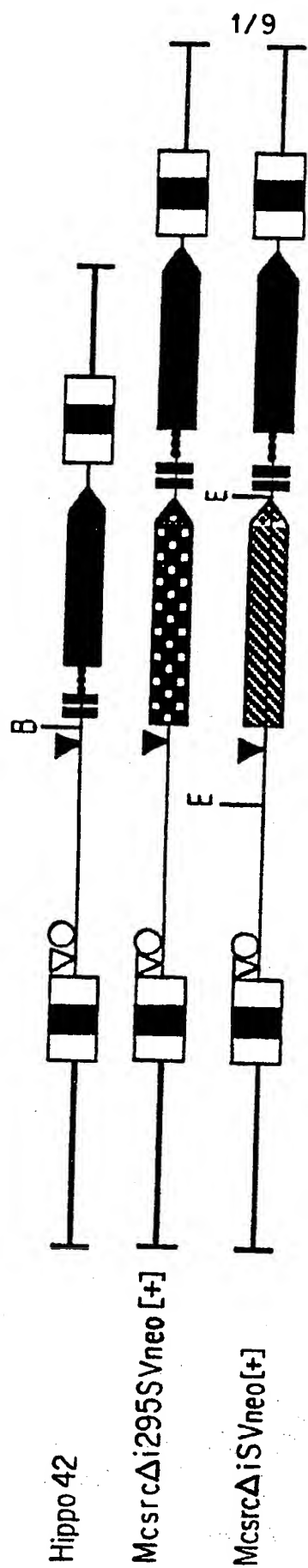
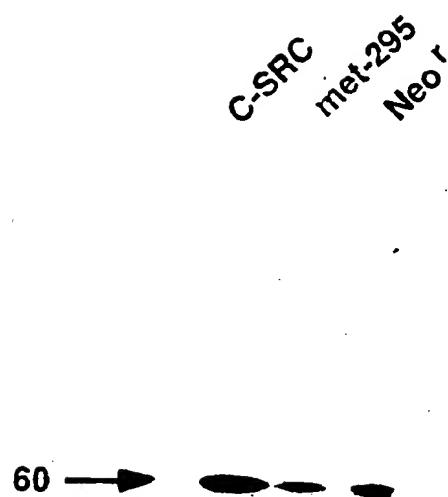


FIG.1

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**FIG. 2**

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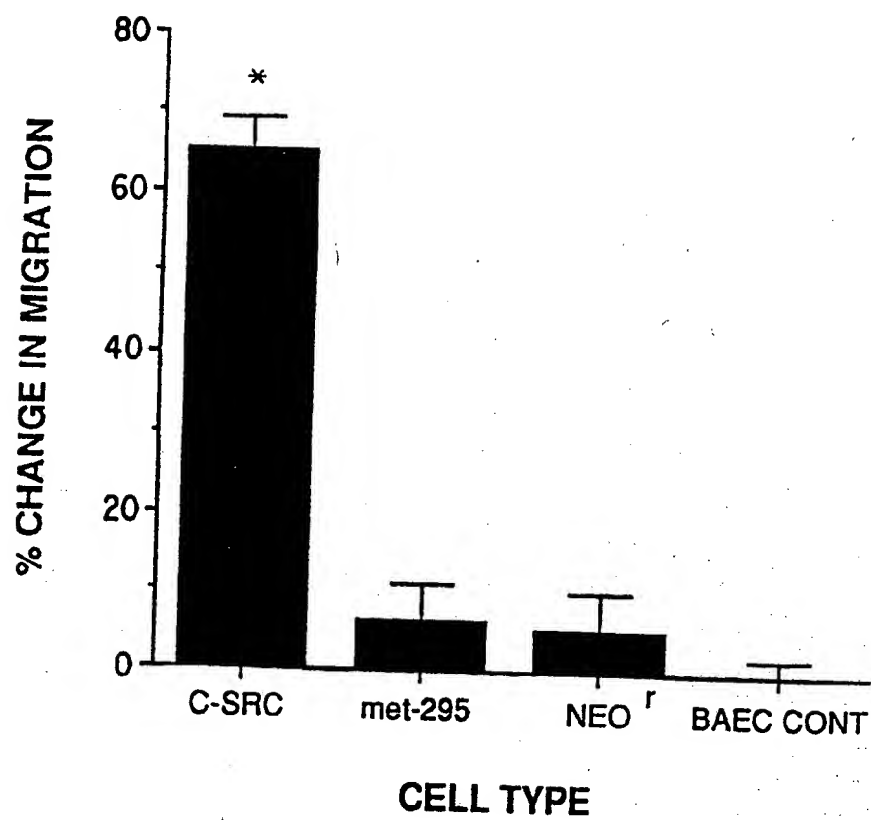


FIG. 3

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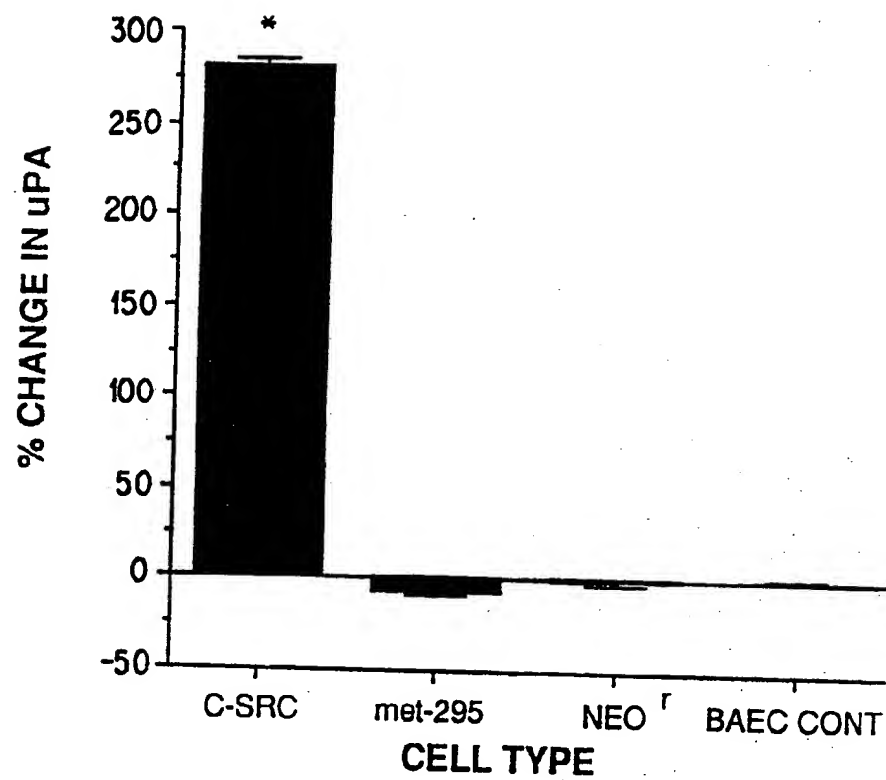


FIG.4

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**FIG. 5A**



**FIG. 5B**

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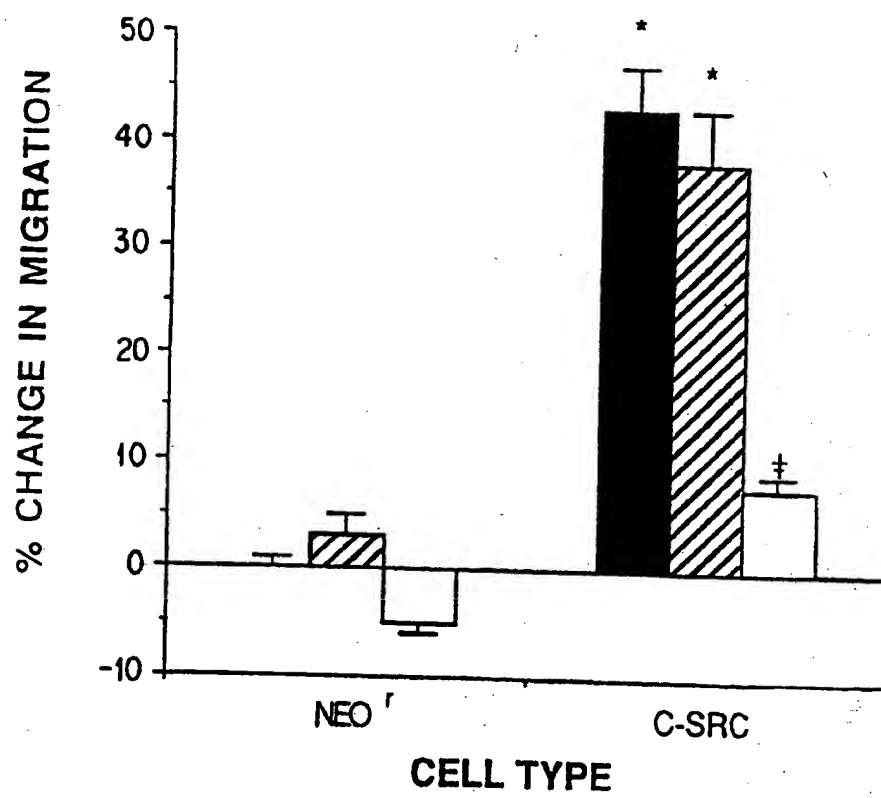


FIG. 6

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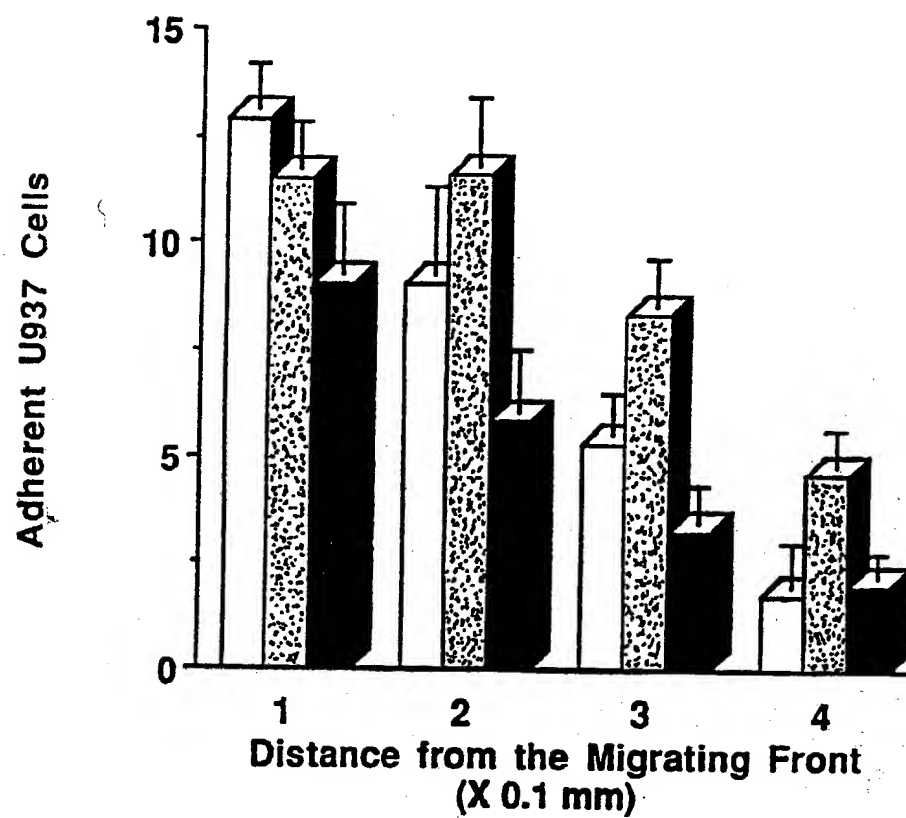


FIG. 7

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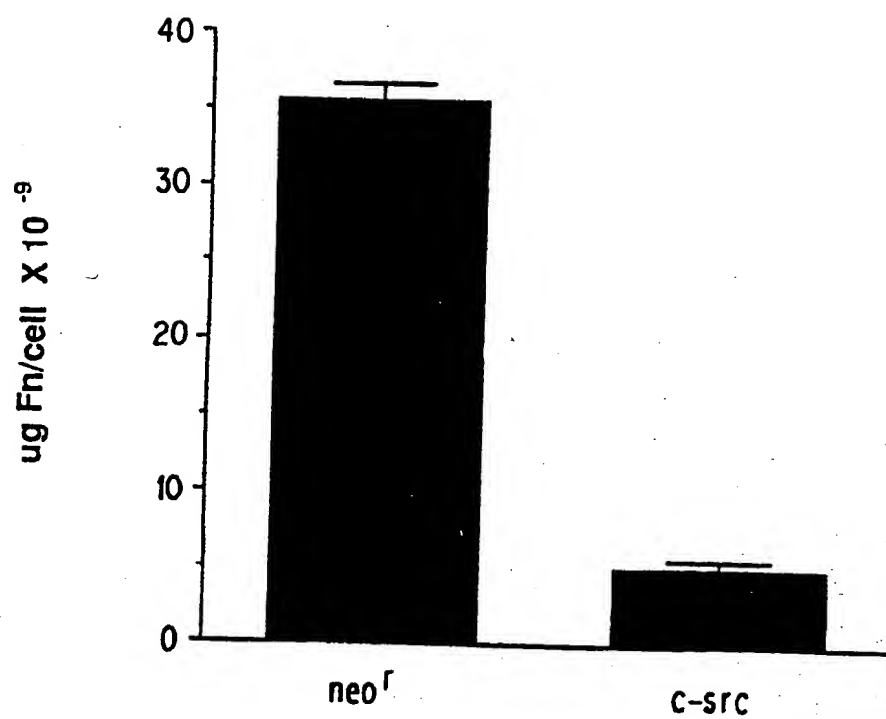


FIG. 8

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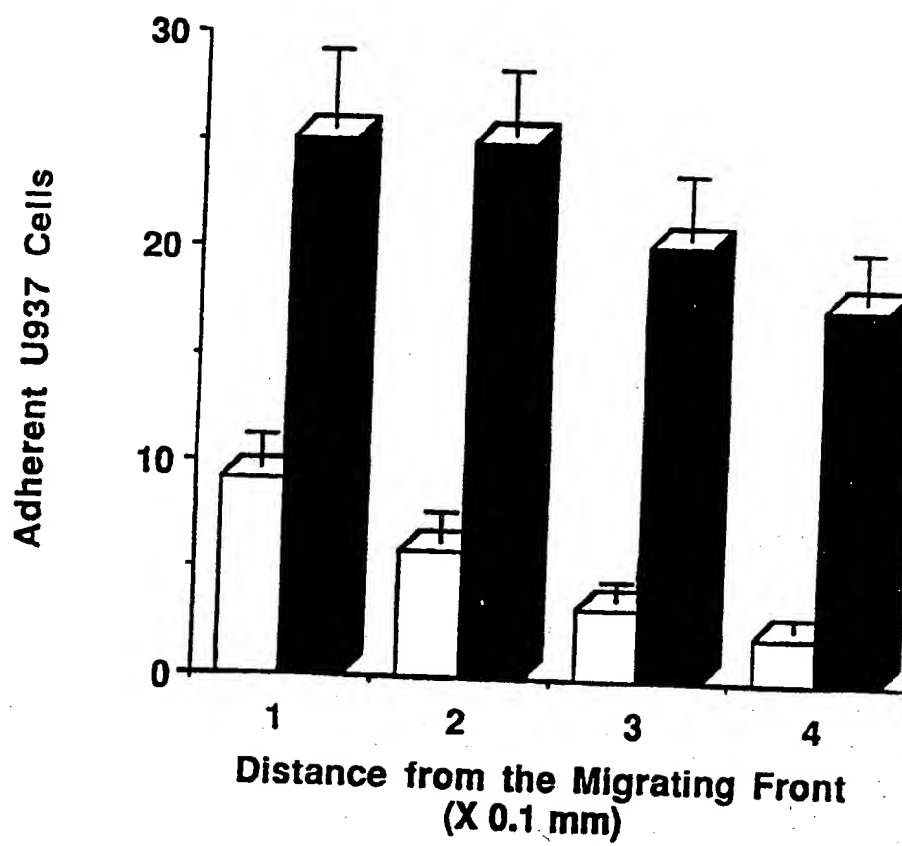


FIG.9

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/00445

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) : C12N 5/16, 5/22, 15/12, 15/85; C07H 15/12; A61F 2/02, 2/06

US CL : 435/240.2, 172.3, 320.1; 536 23.5; 600/36; 623/1, 11

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.2, 172.3, 320.1; 536 23.5; 600/36; 623/1, 11

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 90/06997 (Anderson <i>et al.</i> ) 28 June 1990. See entire document.	1-29
Y	Am. J. Pathol., vol. 137, no. 1, issued July 1990, Bell <i>et al.</i> , "Influence Of Angiotensin System On Endothelial And Smooth Muscle Cell Migration", pages 7-12. See entire document.	1-29
Y	J. Biol. Chem., vol. 265, no. 3, issued 25 January 1990, Bell <i>et al.</i> , "Plasminogen Activator Gene Expression Is Induced By The <i>src</i> Oncogene Product And Tumor Promoters", pages 1333-1338.	1-29
Y	Circulation, vol. 80, no. 5, issued November 1989, Dichek <i>et al.</i> , "Seeding Of Intravascular Stents With Genetically Engineered Endothelial Cells", pages 1347-1353. see entire document.	1-29

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	g	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

01 MARCH 1993

Date of mailing of the international search report

29 MAR 1993

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Authorized officer

CHRISTOPHER S. F. LOW

Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/00445

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Vasc. Surg., vol. 11, issued 1990, Örtengwall <i>et al.</i> , "Endothelial Cell Seeding Reduces Thrombogenicity Of Dacron Grafts In Humans", pages 403-410. See entire document.	1-29
Y	Molec. Cell. Biol., vol. 8, no. 2, Warren <i>et al.</i> , "Elevated Expression Of pp60 <sup>c-src</sup> Alters A Selective Morphogenetic Property Of Epithelial Cells In Vitro Without A Mitogenic Effect", pages 632-646. See entire document.	1-29
Y	Biochim. Biophys. Acta, vol. 948, issued 1988, Perlmutter <i>et al.</i> , "Specialized Protein Tyrosine Kinase Proto-Oncogenes In Hematopoietic Cells", pages 245-262. See entire document.	1-29
Y	Cell, vol. 50, issued 11 September 1987, Jove <i>et al.</i> , "Enzymatically Inactive p60 <sup>c-src</sup> Mutant With Altered ATP-Binding Site Is Fully Phosphorylated In Its Carboxy-Terminal Regulatory Region", pages 937-943. See entire document.	1-29
Y	Proc. Natl. Acad. Sci. USA, vol. 81, issued October 1984, Cone <i>et al.</i> , "High-Efficiency Gene Transfer Into Mammalian Cells: Generation Of Helper-Free Recombinant Retrovirus With Broad Mammalian Host Range", pages 6349-6353. See entire document	1-29
Y	Science, vol. 244, issued 16 June 1989, Nabel <i>et al.</i> , "Recombinant Gene Expression <i>in Vivo</i> Within Endothelial Cells Of The Arterial Wall", pages 1342-1344. See entire document.	1-29

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/00445

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

Automated Patent System: USPAT and JPOABS

DIALOG: files 434, 5, 155, 399, 159, 144, 266, 444,

CD-ROM ENTREZ sequences release 1.0

GENBANK, EMBL, vectorbank 6.4, UEMBL N-GenSeq

Search terms:

endothelial cell(s) transformed transfected graft(s) prosthesis(es) c-src protein dna pp60c-src pp60v-src  
oncogene(s) protooncogene(s)